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## Sex Differences in Vaccine-Specific and Heterologous Immunity Following Administration of Measles and /or DtwP Vaccines to Nine Month-Old Gambian Infants

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**SEX DIFFERENCES IN VACCINE-SPECIFIC AND HETEROLOGOUS  
IMMUNITY FOLLOWING ADMINISTRATION OF MEASLES AND / OR DTwP  
VACCINES TO NINE MONTH-OLD GAMBIAN INFANTS**

**Fatou Noho-Konteh B.Sc M.Sc**

Thesis submitted to Open University, U.K. in fulfilment of the requirements for the  
Master of Philosophy in the field of Life Sciences

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**AFFILIATED RESEARCH CENTRE**

MRC The Gambia, PO Box 273, Atlantic Road, Fajara, The Gambia, West Africa

**COLLABORATING ESTABLISHMENTS**

Division of Pathway Medicine, University of Edinburgh, Scotland, UK; The National  
Institute for Public Health and The Environment (RIVM), Bilthoven, The Netherlands

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*Dedicated to my husband Sisawo Konteh  
and my children, Amie and Sabelle Konteh  
I am blessed to have such a wonderful family*



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## ABSTRACT

The expanded programme of immunization was introduced in The Gambia over 30 years ago, using empirically developed vaccines with limited understanding of exactly how they work. Observational studies showing vaccines have heterologous beneficial or deleterious effects on disease susceptibility, dependent on sex, have been controversial, with no immunological data to support these epidemiological findings. The live measles vaccine (MV) seems to have beneficial protective effects, while diphtheria, tetanus, whole cell pertussis (DTwP) vaccine increases disease susceptibility, both effects being more prominent in females. These effects are modified when the vaccines are given simultaneously.

This thesis describes the results of a randomized trial primarily aimed at studying the heterologous effects of MV and DTwP vaccination of 9 month old Gambian infants. Assays included multiplex cytokine analysis of culture supernatants, flow cytometric intracellular staining, vaccine antibody assays, and whole transcriptome microarray analysis.

Measles and DTwP antibodies were not affected by combining vaccines or by gender. When DTwP is given with MV, the measles-specific response was skewed away from a protective CD8 IFN- $\gamma$  response, while TT responses become more pro-inflammatory suggesting that MV has an adjuvant effect on DTwP immunity.

A striking finding was the immunosuppressive effect of DTwP, particularly in females. There was a reduction in pro-inflammatory cytokines and reduced IFN- $\gamma$ :IL-10 ratios in  $\alpha$ CD3/28 cultures, alongside down-regulated interferon response gene pathways, and increased innate IL-10 production in DTwP vaccinated females. By contrast, DTwP vaccinated males a more pro-inflammatory profile than females with only upregulated genes post-vaccination. Males vaccinated with MV+DTwP were less pro-inflammatory

than females, and had only down-regulated genes suggesting this combination had opposite effects in males and females.

The results provide plausible immunological explanations for the observed beneficial and deleterious effects of MV and DTwP respectively. These will now need to be tested in carefully designed prospective studies.

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**List of Abbreviations**

adenylate cyclase toxin

|                 |  |
|-----------------|--|
| <b>ACT</b>      |  |
| <b>ADCC</b>     | Antibody-dependent cell-mediated cytotoxicity              |
| <b>aP</b>       | acellular pertussis  |
| <b>APC</b>      | Antigen presenting cells                                   |
| <b>ARTN</b>     | Artemin  |
| <b>BCG</b>      | Bacillus Calmette–Guérin                                   |
| <b>BCR</b>      | B cell receptors   |
| <b>BD</b>       | Becton-Dickinson   |
| <b>BSA</b>      | Bovine serum albumin                                       |
| <b>CMI</b>      | Cell-mediated immunity                                     |
| <b>CNS</b>      | Central Nervous System                                     |
| <b>CRFs</b>     | Case report forms  |
| <b>CTL</b>      | Cytotoxic T lymphocytes                                    |
| <b>CTLA4</b>    | cytotoxic T lymphocyte antigen 4                           |
| <b>DC</b>       | Dendritic cells  |
| <b>DMSO</b>     | Dimethyl sulfoxide   |
| <b>DPM</b>      | Department of Pathway Medicine                             |
| <b>DPYSL5</b>   | dihydropyrimidinase-like 5                                 |
| <b>DT</b>       | Diphtheria and Tetanus vaccine                             |
| <b>DTaP</b>     | Diphtheria, Tetanus, acellular Pertussis Vaccines          |
| <b>DTwP</b>     | Diphtheria, tetanus, whole cell pertussis combined vaccine |
| <b>DTx</b>      | diphtheria toxoid  |
| <b>EDTA</b>     | Ethylenediamine tetra-acetic acid                          |
| <b>ELISA</b>    | The enzyme-linked immunosorbent assay                      |
| <b>EPI</b>      | The Expanded Programme of Immunization                     |
| <b>EU/ml</b>    | Endotoxin Units  |
| <b>FACS</b>     | Fluorescence Activated Cell Sorter                         |
| <b>FBC</b>      | Full blood count   |
| <b>Fc RIIIA</b> | Fragment Crystallizable region III-A                       |



|                                |   |
|--------------------------------|---|
| <b>FDC</b>                     | Follicular dendritic cells                      |
| <b>FHA</b>                     | filamentous haemagglutinin                      |
| <b>FIM</b>                     | fimbriae  |
| <b>GA</b>                      | gestational age                                 |
| <b>GLS</b>                     | Generalized Least-Squares                       |
| <b>GMCSF</b>                   | granulocyte-macrophage CSF                      |
| <b>GO</b>                      | Gene Ontology                                   |
| <b>HA</b>                      | haemagglutinin antigen                          |
| <b>HAI</b>                     | haemagglutinin inhibition                       |
| <b>HBV</b>                     | Hepatitis B virus                               |
| <b>Hib</b>                     | Haemophilus influenzae B                        |
| <b>ICS</b>                     | intracellular staining                          |
| <b>IFN-<math>\alpha</math></b> | interferon-a                                    |
| <b>IFN-<math>\gamma</math></b> | Interferon gamma                                |
| <b>IgA</b>                     | Immunoglobulin A                                |
| <b>IgD</b>                     | Immunoglobulin D                                |
| <b>IgE</b>                     | Immunoglobulin E                                |
| <b>IGFBP1</b>                  | insulin-like growth factor binding protein 1    |
| <b>IgG</b>                     | Immunoglobulin G                                |
| <b>IgM</b>                     | Immuogloblin M                                  |
| <b>IL</b>                      | Interleukin                                     |
| <b>IPA</b>                     | Ingenuity Pathway Analysis                      |
| <b>IQR</b>                     | Interquertile range                             |
| <b>ISG</b>                     | interferon-stimulated genes                     |
| <b>iTreg</b>                   | induced T regulatory cells                      |
| <b>JCVI</b>                    | Joint Committee on Vaccination and Immunization |
| <b>KEGG</b>                    | Kyoto Encyclopedia of Genes and Genomes         |
| <b>LLOD</b>                    | lower limit of detection                        |
| <b>LLOQ</b>                    | lower limit of quantitation                     |

|               |  |
|---------------|--|
| <b>LN</b>     | lymph nodes                                |
| <b>LPS</b>    | bacterial Lipopolysaccharide               |
| <b>LT</b>     | Lymphotoxin                                |
| <b>LTi</b>    | lymphoid tissue inducer                    |
| <b>MAB</b>    | maternal antibodies                        |
| <b>MCL</b>    | Markov clustering                          |
| <b>MCV</b>    | mean cell volume                           |
| <b>MDG</b>    | Millennium Development Goal                |
| <b>MHC</b>    | Major histocompatibility complex           |
| <b>MHC-I</b>  | Major histocompatibility complex - Class 1 |
| <b>MHC-II</b> | Major histocompatibility complex - Class 2 |
| <b>MMR</b>    | measles-mumps-rubella                      |
| <b>MMRV</b>   | measles-mumps rubella- varicella           |
| <b>MPLA</b>   | monophosphoryl lipid A                     |
| <b>MR</b>     | measles-rubella                            |
| <b>MR</b>     | mortality rate                             |
| <b>MRC</b>    | Medical Research Council                   |
| <b>MRR</b>    | mortality rate ratio                       |
| <b>MUC6</b>   | mucin 6                                    |
| <b>MV</b>     | measles vaccine                            |
| <b>NCAM</b>   | neural cell adhesion molecule              |
| <b>NK</b>     | Natural Killer cells                       |
| <b>NKT</b>    | Natural killer T cells                     |
| <b>NSE</b>    | non-specific effects                       |
| <b>nTreg</b>  | natural Tregs                              |
| <b>OPV</b>    | Oral polio vaccine                         |
| <b>OR</b>     | Odds ratio                                 |
| <b>PAMP</b>   | pathogen-associated molecular patterns     |
| <b>PCV-13</b> | Pneumococcal conjugate vaccine             |

|                               |   |
|-------------------------------|---|
| <b>PDv</b>                    | pandemic influenza vaccine                              |
| <b>PMT</b>                    | Photomultiplier Tube                                    |
| <b>PPD</b>                    | Purified protein derivative                             |
| <b>PRN</b>                    | pertactin   |
| <b>PRR</b>                    | pattern recognition receptors                           |
| <b>PT</b>                     | pertussis toxin   |
| <b>PTx</b>                    | pertussis toxoid  |
| <b>PWv</b>                    | whole virion-particle vaccine                           |
| <b>RBC</b>                    | red blood cell  |
| <b>RBN</b>                    | robust spline normalization                             |
| <b>RIVM</b>                   | National Institute of Public Health and the Environment |
| <b>RNF213</b>                 | ring finger protein 213                                 |
| <b>ROR</b>                    | Retinoid-related orphan receptor                        |
| <b>RSV</b>                    | respiratory syncytial virus                             |
| <b>RT</b>                     | room temperature  |
| <b>SMOX</b>                   | spermine oxidase  |
| <b>TB</b>                     | Tuberculosis  |
| <b>Tc</b>                     | cytotoxic T cells                                       |
| <b>T<sub>CM</sub></b>         | Central Memory T Cells                                  |
| <b>TCR</b>                    | T-cell receptor   |
| <b>TCT</b>                    | tracheal cytotoxin                                      |
| <b>T<sub>EM</sub></b>         | Effector Memory T Cells                                 |
| <b>TGF-<math>\beta</math></b> | transforming growth factor-b                            |
| <b>Th</b>                     | T helper cells  |
| <b>TIV</b>                    | trivalent inactivated influenza vaccine                 |
| <b>TLR</b>                    | Toll-like receptors                                     |
| <b>TNF</b>                    | tumor necrosis factor                                   |
| <b>TPRA1</b>                  | transmembrane protein, adipocyte associated 1           |
| <b>Treg</b>                   | T regulatory cells                                      |

|                |                                     |
|----------------|-------------------------------------|
| <b>Trl</b>     | T cells with regulatory functions   |
| <b>TT</b>      | tetanus toxoid                      |
| <b>VAS</b>     | Vitamin A supplementation           |
| <b>VEGF</b>    | Vascular endothelial growth factor  |
| <b>VitA</b>    | Vitamin A                           |
| <b>VST</b>     | variance stabilizing transformation |
| <b>WHO</b>     | World Health Organization           |
| <b>WHO GHO</b> | WHO Global Health Observatory       |
| <b>wP</b>      | whole cell pertussis                |
| <b>YF</b>      | Yellow Fever                        |

## **CHAPTER 1**

### **INTRODUCTION**

1.1 Infectious Disease Burden in Infants

According to World Health Organization (WHO) estimates, about 20% of all deaths occur in children under 5 year old (WHO) (1996b). In 2011, approximately 7 million children under the age of five died, which amounts to about 800 children dying every hour globally (Figure 1.1) ((WHO), 2012a). This is a reduction from the 12 million deaths reported for 1990. The highest under five death rates occurred in the WHO African Region with 106 deaths per 1000 live births; which is about 8 times higher than that observed in the WHO European region (Figure 1.2) ((WHO), 2012a).

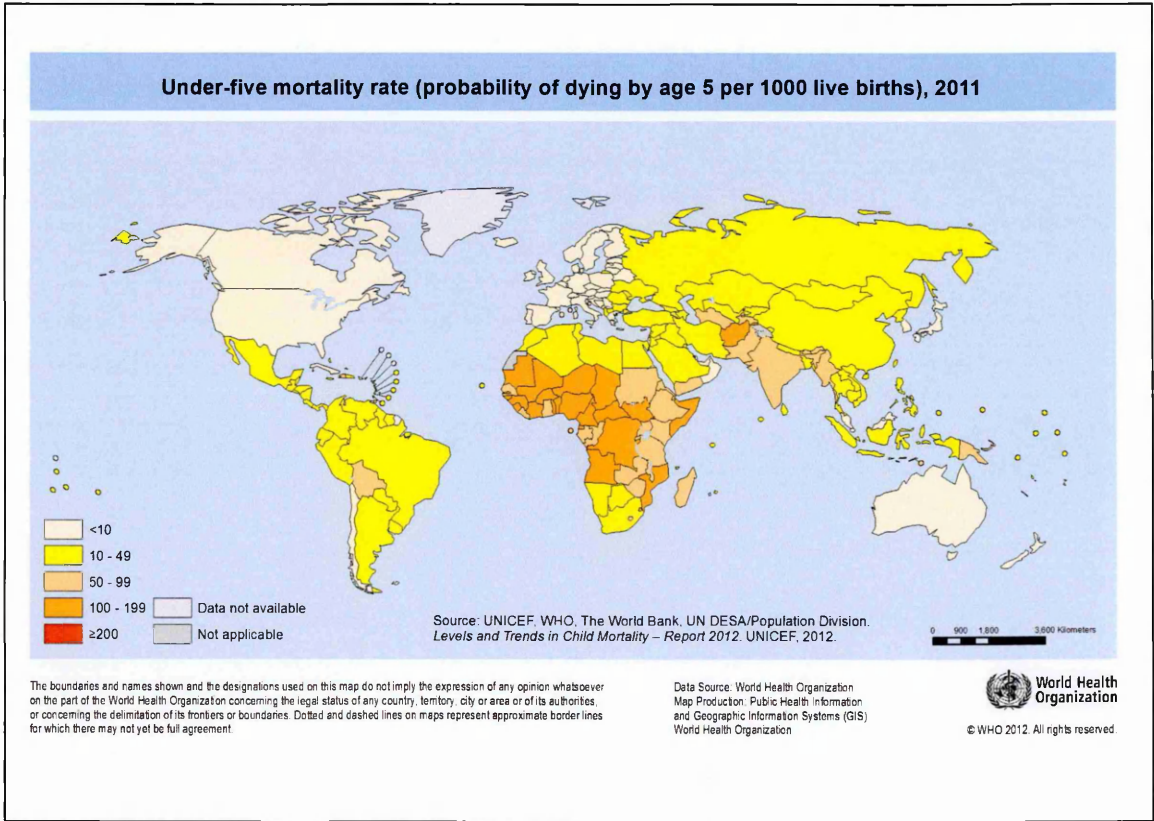
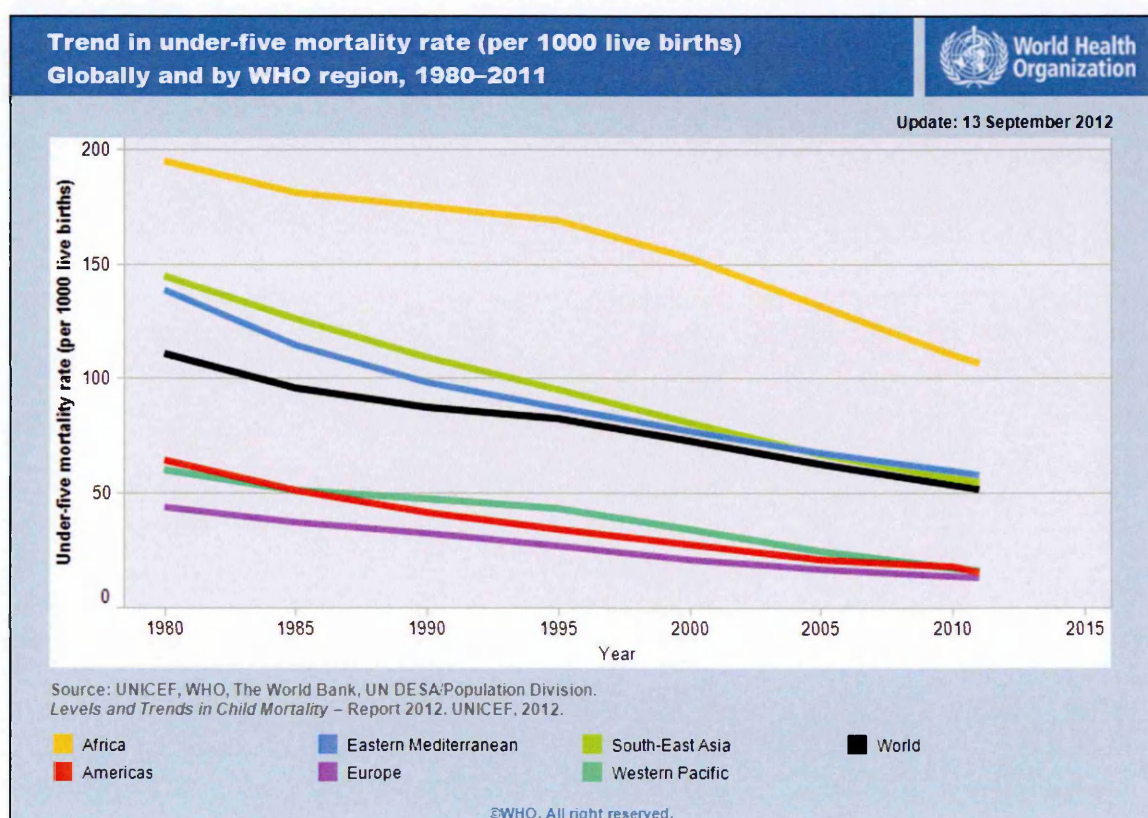
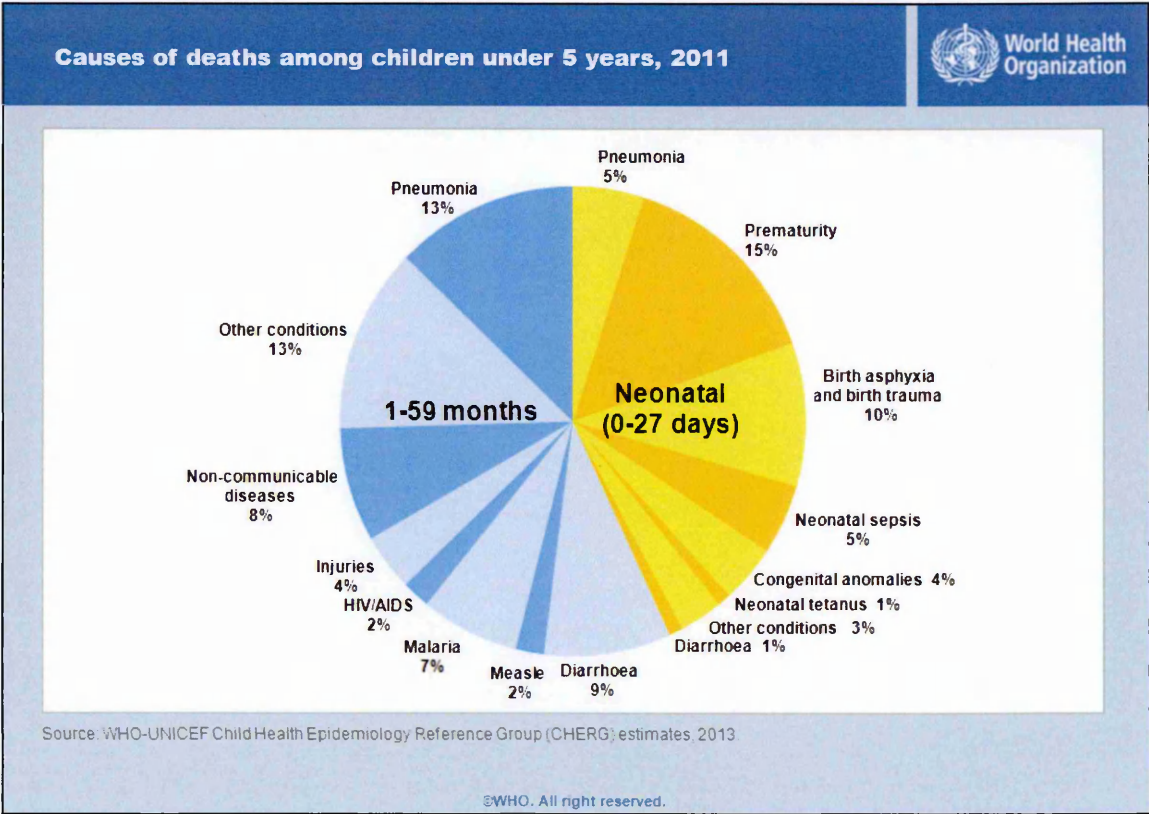


Figure 1.1 Under-five mortality rate for the year 2011.



**Figure 1.2 Global trend in under-five mortality rate for the period 1980 - 2011**

Deaths in infants aged 1 to 6 months are mostly caused by acute respiratory infections (e.g. respiratory syncytial virus (RSV), *Bordetella pertussis*, *Haemophilus influenzae* B (Hib), *Streptococcus pneumoniae* or diarrhoeal diseases (e.g. rotaviruses, *Salmonella* spp., *Shigella* spp.) (Siegrist, 2007). The major causes of death in infants aged 1 to 56 months from 2000 to 2011 were due to pneumonia (13%), diarrhoea (9%), non-communicable diseases (8%) and other infectious and parasitic diseases accounting for 13% (Figure 1.3) ((WHO), 2012a).



**Figure 1.3 Worldwide causes of death among children under-five years old for the year 2011.**

Before the introduction of measles vaccine, measles was the leading cause of death in children worldwide. For example, in the western pacific region, measles reported cases has declined for 3, 382, 000 in 1974 (pre-immunization) to about 146,750 in 2008 (post-immunization) ((WHO), 2004).

**1.2 Introduction of The Expanded Programme of Immunization (EPI) by WHO**

The greatest public health intervention of the twentieth-century was the introduction of The Expanded Programme of Immunization (EPI) by the World Health Organization in 1974. This has greatly reduced infectious disease burden in children worldwide. Between 1900 and 1973, the use of available vaccines was largely confined to industrialized countries, particularly the higher classes (Clements, 1996). Diphtheria, and whole cell pertussis vaccine were introduced in 1923 and 1926 respectively, tetanus toxoid (TT) and BCG in 1927 while yellow fever vaccine became available in 1935 (André, 2003). Other vaccines were introduced after the second world war which are still being used today including the



inactivated and oral polio vaccines introduced in 1955 and 1958 respectively, and the live, attenuated measles, mumps and rubella (MMR) vaccine which became available in 1971 (André, 2003). Gradually, these vaccines were made available to developing countries.

Currently, it is recommended that children are all immunized against diphtheria, tetanus, pertussis, hepatitis B virus, *H. influenzae* type b, poliovirus, *Streptococcus pneumoniae*, rotavirus, measles, mumps, rubella, TB and yellow fever in endemic areas ((CDC), 2011; (WHO), 2011). EPI was implemented in The Gambia in 1979 where there is consistently reported high immunization coverage. Immunization coverage in one year olds was reported as above 95% in 2009 for measles, yellow fever and the third dose of DTP, Hib, HBV and polio ((WHO)-UNICEF, 2011). The current full routine immunization schedule for The Gambia is shown in Table 1.

**Table 1.1 Current EPI vaccine schedule in The Gambia**

| Timing  | Vaccinations   |
|---|--|
| Birth   | BCG, first dose of polio (OPV), first dose of HBV          |
| 2, 3 and 4 months   | DTwP/Hib/HBV combined; third, fourth dose of polio; PCV-13 |
| 9 months  | Measles (MV), yellow fever (YF), fifth dose of polio       |
| 16 months   | DTwP/Hib/HBV combined                                      |
| 18 months   | Sixth dose polio   |
| Plus vitamin A every 6 months (from 6 months of age till 59 months) |  |

**Key**

|        |  |
|--------|--|
| BCG    | Bacillus Calmette Guerin                                       |
| OPV    | Oral polio vaccine   |
| HBV    | Hepatitis B virus  |
| DTwP   | Diphtheria, tetanus with whole-cell pertussis combined vaccine |
| Hib    | Haemophilis influenza B  |
| VitA   | Vitamin A  |
| MV     | Measles Vaccine  |
| YF     | Yellow Fever   |
| PCV-13 | Pneumococcal conjugate vaccine                                 |

*Adapted from (Payne et al., 2013).*

*DTwP/Hib/HBV vaccine is a Pentavalent vaccine*

**1.3 The Value of Infant Vaccination**

Vaccination has been a key player in reducing morbidity and mortality caused by infectious agents. Vaccines work by presenting pathogen antigens to the immune system, which responds by evoking an immune response that eliminates the effects of an infectious pathogen or a disease process. The antigenic material can either be live attenuated pathogens, killed or inactivated forms of these pathogens, or purified or recombinant material such as proteins (Olesen et al., 2009). The aim of vaccination is the induction of long-lasting protective immune memory that is able to respond quickly upon infection with the vaccine specific pathogens. This effect is elicited by the adaptive arm of

the immune system. The innate arm of the immune system is also activated by vaccines and is characterized by limited specificity, but is now thought to influence the magnitude, quality and duration of the adaptive responses (Palm and Medzhitov, 2009). Antibody levels may persist for many years following immunization of adults and older children, but levels wane more rapidly after infant immunization. The mechanisms behind the persistence of antibody are not well understood (Crotty et al., 2003; Kelly et al., 2005; Amanna et al., 2007).

Success stories of vaccination include the eradication of smallpox from the world, the almost complete elimination of poliomyelitis, and a decrease of more than 95% in the incidence of diseases such as diphtheria, tetanus, pertussis, measles, mumps and rubella (Rappuoli et al., 2002).

The current challenge for neonatal vaccination is to develop, and promote at a global level, vaccines that can be safely administered soon after birth and are effective after one or two early doses. This is a difficult task given the immaturity of the immune system in early life (Siegrist, 2001).

Many notable, highly effective vaccines have been designed empirically, with limited understanding of how and why they work. The recommended schedule is based on studies of sero-conversion and protection, and on assumed feasibility of the schedule (Ramsay et al., 1993). There is therefore a need to understand how commonly used childhood vaccines work and how the immune system responds to them more generally. This is the subject of this thesis and will become of increasing importance as vaccine preventable diseases decline and newer generation vaccines are introduced.

#### **1.4 The Immune System**

There are two main branches of immunity; the innate immune system, which is non-specific, and the adaptive immune system, which has specificity (Reviewed in (Kaufmann

and Kabelitz, 2010)). Innate immune elements include phagocytic cells (neutrophils, monocytes, macrophages), granulocytes that release inflammatory mediators (basophils, mast cells and eosinophils), natural killer (NK) cells, NK T cells, dendritic cells, complement, cytokines, and acute phase proteins (Figure 1.4). These function as the first line of defense against foreign antigen (Delves and Roitt, 2000). The adaptive immune response involves antigen-specific reactions mediated through T and B lymphocytes.

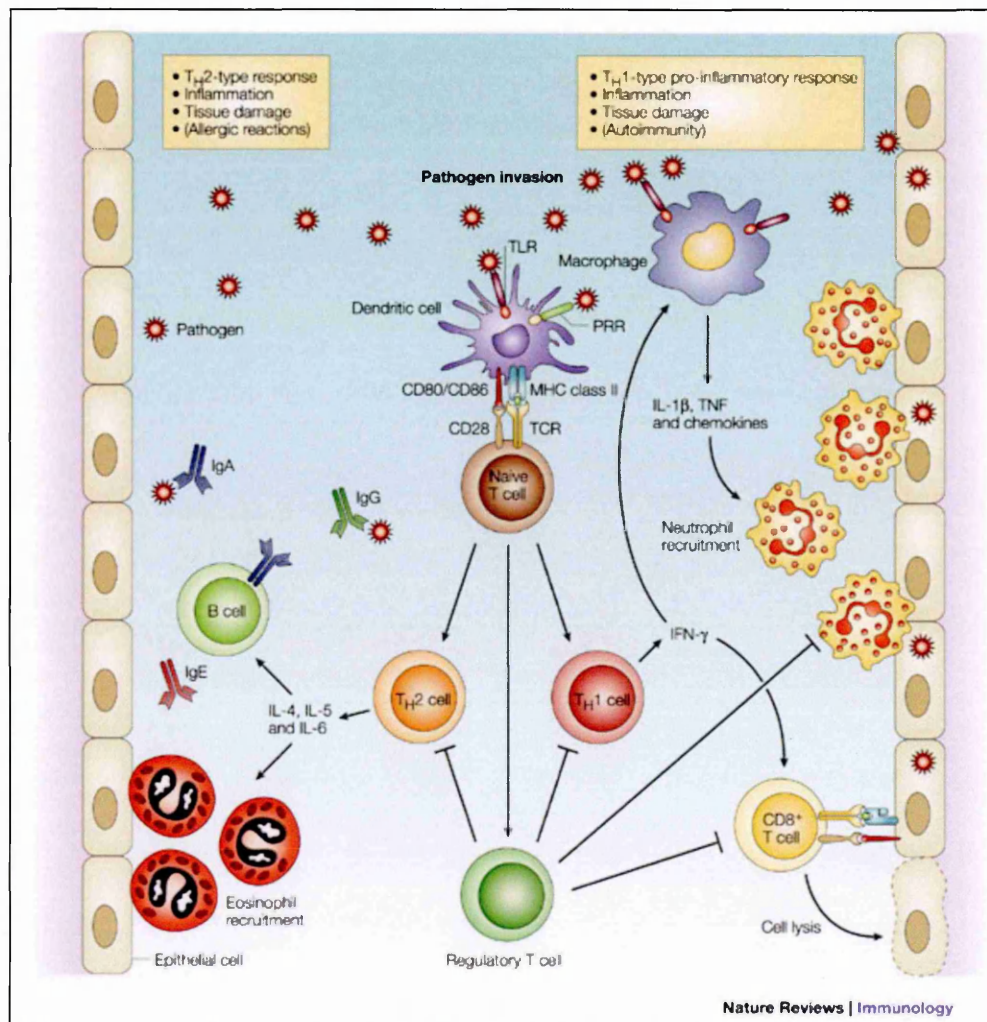
The innate immune response is rapid and immediate while the adaptive immune response evolves more slowly (days or weeks). Adaptive immunity has memory and remembers past encounters with a pathogen and hence the response to subsequent encounters is stronger and more rapid than the initial response (Delves and Roitt, 2000). There is increasing evidence that the various components of the innate immune system exhibit memory characteristics, despite initial assumptions that innate immunity was a fixed entity with no memory (Netea et al., 2011).

#### **1.4.1 Innate Immunity**

Innate immunity evolved earlier than the adaptive immune system and is mediated by cells such as macrophages, NK cells and dendritic cells (DC) (Wan and Flavell, 2009). Dendritic cells (includes the interdigitating dendritic cells of lymph nodes, veiled cells in the blood, and Langerhan's cells in the skin) are professional cells and act as antigen presenting cells (APCs) upon activation. They possess pattern recognition receptors (PRRs), which include the Toll-like receptors (TLRs), on their surface that recognize specific pathogen-associated molecular patterns (PAMPs) on the surface of the pathogen (Figure 1.4) (Medzhitov and Janeway Jr, 1997; Parkin and Cohen, 2001; Mills, 2004).

Research during the past 10 years has identified a major role for the innate immune system in programming protective immune responses to vaccines and adjuvants via different innate signals such as Toll-like receptors (Pulendran and Ahmed, 2011). The innate immune system can instruct the adaptive immune response (Janeway and

Medzhitov, 2002). The neonate possesses a functionally distinct innate immune system, including a bias against Th1-cell-polarizing cytokines, thus contributing to a distinct pattern of neonatal adaptive immune responses (Levy, 2007; Kollmann et al., 2012).



**Figure 1.4 Innate immune effector cells**, including macrophages, dendritic cells (DCs), neutrophils and natural killer (NK) cells (not shown), together with various protein components of the complement system; provide the first line of defense against invading microorganisms. Mills K.H. G. et al. 2004

#### 1.4.1.1 Natural Killer cells

Natural killer (NK) cells are large granular lymphocytes of the innate immune system. NK cells develop in the bone marrow from the common progenitor cell and circulate in the blood. NK cells are innate immune effectors that, by cytokine production or cytotoxicity, help to contain an infection until an effective adaptive response is mounted. They are distinct from T and B cells in that they are controlled by a limited repertoire of germ line-

encoded receptors that do not undergo somatic recombination (Lanier, 2005). They were later recognized as a separate lymphocyte lineage, with both cytotoxicity and cytokine-producing effector functions. These cells constitute the third major lymphocyte subset and represent approximately 10–15% of circulating lymphocytes in blood. NK cells play an important role in host immunity because they have the ability to quickly mediate cellular cytotoxicity against pathogen-infected or malignantly transformed cells and to produce a wide variety of chemokines and cytokines that influence other cellular compartments of the immune system. They have a limited life-span *in-vivo* and hence must be continually replenished to maintain homeostasis (Yokoyama et al., 2004).

NK cells have a multitude of inhibitory and activating receptors that engage MHC class I molecules, MHC class I-like molecules, and molecules unrelated to MHC. NK cells are restricted in what target cells they can engage by the expression of the target's MHC ligands, but in a very complex fashion that remains incompletely understood (Ljunggren and Karre, 1990). CD8 T cells and NK cells complement each other in that they both have a role in target recognition and host defense, and their similar mechanisms of cytolysis, suggesting that these 2 cell types may have each evolved from a common ancestral cytolytic effector cell (Sun and Lanier, 2011). The killing mechanism uses direct exocytosis of secretory lysosomes that contain lytic proteins such as perforin, granzymes, and Fas ligand (Trapani and Smyth, 2002; Bossi and Griffiths, 2005). NK cells may instruct and shape adaptive immune responses through the release of cytokines (Biron et al., 1999; Shi et al., 2000) or by direct interaction with dendritic cells (Munz et al., 2005; Zitvogel et al., 2006).

#### **1.4.1.2 Natural Killer cell Receptors**

The cell surface phenotyping most commonly used for defining human NK cells within the lymphocyte gate on the flow cytometric analyzer shows an absence of CD3 (thereby excluding T cells and NKT cells which express CD3 on their surface) and expression of CD56 (Trinchieri, 1989), the 140-kDa isoform of neural cell adhesion molecule (NCAM)

found on NK cells and a minority of T cells (Ritz et al., 1988; Lanier et al., 1989). NK cells may also express CD44, CD11a and CD16 on their surface (Cooper et al., 2001; Lima et al., 2002).

## **1.5 Adaptive Immunity**

Adaptive immunity evolved after innate immunity and is mediated by T cells and B cells (Wan and Flavell, 2009) in order to enhance pathogen eradication by providing antigen specificity and memory onto pre-existing innate immunity. Adaptive immunity is defined by the antigen-driven differentiation of clonally restricted lymphocyte precursors into effector cells with enhanced functional potential (Wan and Flavell, 2009). The adaptive arm of the immune system is divided into B cell and T cell compartments. The B cell functions can be further divided into different functional and memory populations and also according to the secretion of different classes of antibodies. CD4 and CD8 T cells have different effector functions and can be identified by the co-expression of the cell-surface protein CD3, with either CD4 or CD8 respectively. CD4 is expressed on the surface of a variety of T cell subsets with diverse functions (Th1, Th2, Th9, Th17, Th22, Tregs). CD4 T cells recognize peptide in association with MHC class II molecules (Kenneth et al., 2008). CD8 is expressed by cytotoxic T-cells and this cell type can act against viruses and other intracellular pathogens via specific recognition of peptides bound to MHC class I molecules on the surface of the infected cells by their T cell receptors (TCRs). This leads to apoptosis and killing of target cell or release of cytokines with antiviral properties that subsequently lead to viral clearance (Whitton and Fujinami, 2001). Apart from their antiviral activities, CD8<sup>+</sup> T cells also have the ability to protect against intracellular bacteria and tumours (Best et al., 2013).

### **1.5.1 B cells**

B cells develop in the bone marrow from progenitor cells and reside in the marrow for the entire length of their development. Antigen-specific receptors, the B cell receptors (BCRs), are formed by gene rearrangement and recombination events, which are important for

generating a diverse repertoire of the BCR (Parkin and Cohen, 2001). These B cells constitute about 15% of all cells circulating in peripheral blood and their main function is the production of immunoglobulins (Chaplin, 2010). B cells produce five immunoglobulin (Ig) classes, namely, IgM, IgD, IgG, IgA and IgE. These antibodies have various functions such as neutralizing (toxin) effects, binding to mucosal surfaces and hence preventing organisms from binding to the surface, activation of complement, opsonization of bacteria for phagocytosis, and sensitization of tumor and infected cells for antibody-dependant cellular cytotoxicity (ADCC) and subsequent killing (Kaufmann and Kabelitz, 2010). Antibodies therefore augment elements of the innate immune system.

#### **1.5.1.1 Antibody Classes**

When an antigen binds to a B cell receptor (BCR) on the surface of the B cell, the cell proliferates and differentiates into a plasma cell. The plasma cell is the effector form of B lymphocytes and produces antibodies, which are a secreted form of the BCR and have identical antigen specificity. Antibodies are immunoglobulins (Ig). There are five different classes of immunoglobulins, IgM, IgD, IgG, IgA and IgE. They can be distinguished by their C-region. In humans, IgG can be divided into four subclasses namely IgG1, IgG2, IgG3 and IgG4, and IgA antibodies have two subclasses called IgA1 and IgA2. The IgG subclasses are named in order of their abundance in serum with IgG1 being the most abundant (Kenneth et al., 2008). The abundance of antibodies is variable depending on the site, for example, IgM is more abundant in the vascular system while IgG is the predominant antibody found in blood and tissues, and IgA predominates in the mucous membranes of the respiratory and gastrointestinal tracts (Parkin and Cohen, 2001).

#### **1.5.1.2 Role of Neutralizing Antibodies in vaccination**

The hallmark of an effective vaccine response is regarded as the presence of persisting antibodies following vaccination. The antibody response is the first line of the adaptive memory component of host defense against infection (Siegrist, 2008; Plotkin, 2010). The EPI vaccines can induce defined protective serum antibody titres which can be measured



by an enzyme-linked immunosorbent assay (ELISA), or by haemagglutination or neutralization assays, and these serve as markers of vaccine efficacy (Plotkin, 2010). Antibodies can neutralize viruses that infect humans or animals in a number of ways. For example, they may interfere with virion binding to receptors, block virus uptake into cells, prevent uncoating of the viral genome in endosomes, or cause aggregation of virus particles (McLain et al., 1995; Parren and Burton, 2001). Furthermore, induction of lysis of enveloped viruses can occur when antiviral antibodies and serum complement disrupt membranes (Burton, 2002).

#### **1.5.1.3 Development of immunity in early life**

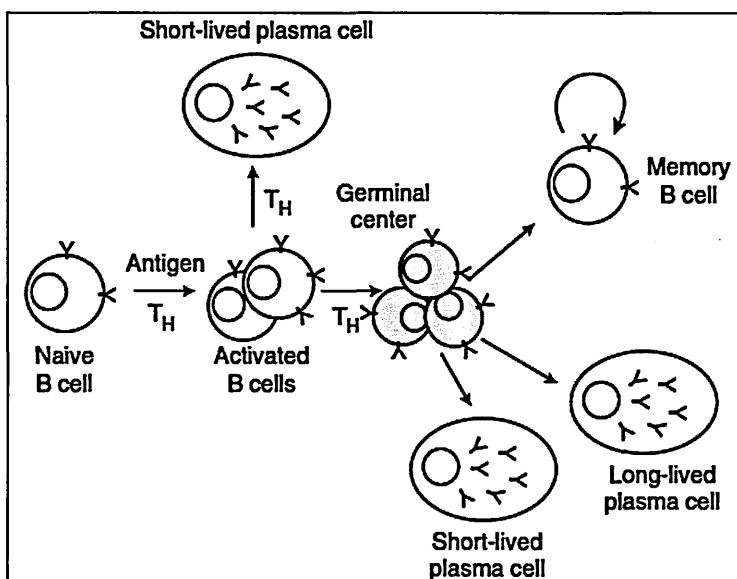
The foetal immune system is Th2 skewed, most likely as a strategy to avoid triggering an alloimmune Th1-type responses to maternal tissues that might lead to premature birth or spontaneous abortion (Philbin and Levy, 2009). At birth, babies are no longer protected by the sterile environment of the womb and are exposed to foreign antigens in the outside environment, for example in the air they breath and on their skin. The neonatal immune system is therefore required to develop protective mechanisms to combat these challenges.

Newborns rely heavily on their innate immune system for protection when they first encounter foreign antigens (Adkins et al., 2004). Neonatal monocytes and antigen presenting cells (APCs) have reduced capability in producing Th1 cytokines such as TNF- $\alpha$  and IFN- $\gamma$  upon antigenic stimulation. Both of these cytokines are important for protection against intracellular pathogens and are important for the development of the subsequent adaptive immune system. This reduced response however is dependent on the type of antigenic stimuli; some of which can induce neonatal APCs adequately (Reviewed in (Burl et al., 2011)). The Burl study found reduced Th1 polarizing cytokine responses in cord blood, with the highest response obtained with TLR8 agonist stimulation at birth. In the same study, proinflammatory cytokine responses were enhanced by 1 month of age and were more pronounced in response to TLR4, -5 and -8

agonists, but this changed with time up to the 1 year of age (Burl et al., 2011). These TLRs are well developed and fully functional in newborns. In healthy infants, TLR expression and signaling up to the first 5 years of life is similar to adult levels in mononuclear cells (Kollmann et al., 2012). The immune system of term infants is intrinsically Th2 skewed, have high levels of the anti-inflammatory cytokine IL-10, and the Th17 supporting cytokines IL-6 and IL-23. They then rapidly acquire antiviral (IFN- $\alpha$ ) and pro-inflammatory (TNF- $\alpha$ , IL-1 $\beta$ ) innate responses as the IL-10, IL-6 and IL-23 responses wane (Angelone et al., 2006; Kollmann et al., 2012). IL-6 is believed to provide protection at birth by inducing an acute, initial response against bacterial infections and gets rid of microbial products and PRR agonists (Angelone et al., 2006).

#### **1.5.1.4 Development of the B cell response in early life**

It has long been established that infants do not mount adequate antibody responses to polysaccharide vaccines against *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*. Indeed, at birth the infant is better able to respond to protein antigens than glycoproteins and polysaccharides (Glezen, 2001). Induction of humoral responses to vaccines is initiated in the germinal centre of lymphoid follicles by follicular dendritic cells (FDC). FDCs play a central role in the activation of B cell differentiation into plasma cells or memory B cells for antibody production (Figure 1.5) (Lambert et al., 2005; Amanna et al., 2006). The poor antibody response to these antigens has been attributed to a delayed maturity of germinal centres in human neonates since the number of mature germinal centres increases with age of the infant with germinal centres first becoming apparent about 4 months after birth (Pihlgren et al., 2003; Adkins et al., 2004; Kruschinski et al., 2004).



**Figure 1.5 Model of B-cell differentiation during an antiviral immuneresponse**  
Following antigenic stimulation, naive B cells undergo clonal expansion and form clusters of activated B cells known as extra-follicular foci. These activated B cells can either differentiate into short-lived plasma cells, or they can migrate into the follicle and initiate a germinal centre reaction. After proliferation and affinity maturation, germinal centre B cells produce both short- and long-lived plasma cells that produce high affinity antibodies, and memory B cells that have high affinity B-cell receptors (Amanna IJ et al., 2006).

The antibody response to polysaccharide vaccine antigens is T cell-independent and takes place in the marginal zone of the spleen. However, infant spleen histological studies have revealed that the marginal zone does not reach full development until two years of age, probably accounting for the delayed antibody response to these encapsulated bacteria (Zemlin et al., 2007). Despite this impaired primary antibody response, some neonatal B cells in germinal centers differentiate into memory B cells.

#### 1.5.1.5 Development of the antibody response in early life

Humoral immunity can be acquired in response to a vaccine or any other antigen encountered in life. The response on second encounter with the same antigen is larger in magnitude and quality, and occurs more rapidly than during the primary encounter; this is the basis for the success of vaccination (Murphy et al. (2008). IgM is the first antibody secreted after encountering an antigen, followed by IgG, IgA or IgE after class switching (Pan-Hammarstrom et al., 2007). *In utero* infants make some immature low affinity IgM but at birth it is maternally derived IgG that dominates in the neonate. As IgM levels rise

on antigen encounter and maternal IgG levels decline, infant derived IgM becomes the dominant antibody at about 3 months of age (Reviewed in (Ygberg and Nilsson, 2012)).

#### **1.5.1.6 Maternal antibodies and inhibition of infant vaccine antibody responses**

Newborn infants transplacentally acquire IgG antibodies from their mothers that are important for protection against infections that occur in the first months of life. There is variability in both the level and rate of waning of maternally acquired antibodies (MAbs). The antibody levels wane over the first 6 months of life and are usually gone by 1 year of age. IgG1 isotype is the most efficiently transferred transplacentally to the foetus followed by IgG4, IgG3 and IgG2 (Reviewed in (Palmeira et al., 2012)). For example, the main antibody subclass that protects against encapsulated bacteria such as *H. influenzae b* is IgG2. Since IgG2 is rarely transported across the placenta to the fetus, newborns are not protected from this pathogens until 4 – 5 months of age when they start to develop their own antibodies. The other immunoglobulin subclasses including IgM are unable to cross the maternal-placenta interface and therefore any present at birth is derived from the infant.

Human breast milk is also a good source of protective maternal antibodies for the neonate. It contains a high concentration of secretory IgA and IgG together with cytokines, antibacterial peptides and other immune cells (Brandtzaeg, 2008).

In addition to protecting against infections, maternal IgG antibodies also affect the infant's response to immunizations. Thus MAb is thought to be capable of inhibiting infant vaccine responses, as initially described for the live measles vaccine (Albrecht et al., 1977) and oral poliomyelitis vaccine (Perkins et al., 1959). Inhibition has also been reported for non-live vaccines such as pertussis (Burstyn et al., 1983; Englund et al., 1995), tetanus and diphtheria toxoids (Claesson et al., 1989; Bjorkholm et al., 1995), Hib conjugate vaccine (Claesson et al., 1989; Daum et al., 1991) and hepatitis A vaccine (Kanra et al., 2000). However it should be borne in mind that other studies reported no influence of MAb on

responses to the same vaccines (Gans et al., 1998; Siegrist et al., 1998a; Siegrist et al., 1998b; Gans et al., 1999).

The practice of administering repeated doses of vaccines such as for DTP, polio, Hib or pneumococcal vaccines, is often sufficient to overcome the inhibition by MABs (Sarvas et al., 1992). The titre of MAB present at the time of immunization is the main determinant of the MAB-mediated inhibition of antibody responses in neonatal mice and humans (Markowitz et al., 1996; Gans et al., 1998; Siegrist et al., 1998a; Siegrist et al., 1998b; Gans et al., 1999). The hypothesis for MAB inhibition is that introduction of vaccine antigen into an infant with pre-existing passive antibodies results in the formation of antigen-antibody complexes. MABs bind to specific B-cell vaccine epitopes, preventing infant B cells from having access to such epitopes; the importance of this inhibitory influence depends upon the vaccine antigen / MAB ratio (Siegrist, 2003).

Recommendations for measles immunization have traditionally been based on the age of MAB disappearance for the majority of infants (Williams et al., 1995), thus it is given at 9 months of age in many developing countries including The Gambia, although more recently WHO have recommended deferring it to 12 months of age in countries with low rates of transmission ((WHO), 2009a). This strategy however, fails to prevent early cases of infection, which are often the most severe. In relation to this, a randomized trial by Aaby and colleagues found that when the first dose of MV was given at 4.5 months in addition to MV at 9 months, there was a 94% vaccine protective efficacy against measles during an outbreak, with 100% protection against admission to hospital and death due to measles (Martins et al., 2009).

#### **1.5.1.7 Role of humoral immunity in vaccination induced protection**

The majority of commonly used childhood vaccines were developed empirically, with the intention of inducing protective antibodies. Indeed, almost all available licensed vaccines elicit a robust antibody response that correlates with the level of protection elicited by the

vaccine (Plotkin, 1999; Plotkin, 2001). Neutralization by antibodies can occur on mucosal surfaces during replication, in an extracellular state after passing through the bloodstream or in a transient extracellular phase (Plotkin, 1999; Plotkin, 2001).

It was believed in the last years of the 20<sup>th</sup> century that the cell mediated immune response confers protection to intracellular pathogens, while protection against extracellular pathogens is dependent on antibody-mediated responses. It is now believed that antibody-mediated immunity is also responsible for the protection induced by most viral vaccines as well (Moore et al., 2003), with the exception of certain chronic intracellular infections, for example *Mycobacterium tuberculosis*, where a CMI response is central to protection.

Humoral immunity has a critical role in protection against measles, which is demonstrated by the protection conferred to infants by maternal antibodies acquired through the placenta (Albrecht et al., 1977). Neutralizing antibodies are elicited by measles virus haemagglutinin and fusion surface glycoproteins, and studies in humans have demonstrated that at least 120 mIU/mL of neutralizing antibody are necessary for protection (Chen et al., 1990). The proportion of infants who develop protective antibody titres when given measles vaccine at 9 months of age is estimated at 85%, while 90%–95% will respond when measles vaccine is given at 12 months of age.

### **1.5.2 T cells**

The human thymic epithelium and medulla develop from the third pharyngeal pouch and cleft complex. Haematopoietic stem cells start to enter the thymus as early as gestational age (GA) 8 weeks. By GA 20 weeks, the thymus is fully developed (Reviewed in (Ygberg and Nilsson, 2012)). T cell development in the thymus has already started by GA 16 – 20 weeks and T cells begin to enter the blood stream at this time to form the peripheral T cell pool. There is a continuous migration of T and B cells as well as monocytes after birth into

lymphoid tissue with increasing age and with exposure to new antigens (Reviewed in (Hoorweg and Cupedo, 2008)).

T cells are classified into specific subpopulations based on the expression of cell surface molecules, as mentioned previously. CD4 and CD8 T cells form the majority of T cells. Most of these express a T cell receptor (TCR) made up of the  $\alpha\beta$  chain heterodimer and are referred to as conventional T cells. Other T cell types include those expressing a TCR consisting of  $\gamma$  and  $\delta$  chains called the gamma-delta ( $\gamma\delta$ ) T cells and NKT cells.

#### **1.5.2.1 T cell immunity in the newborn**

The newborn child has intrinsically Th2 biased immunity and suboptimal APC function (IFN- $\gamma$  production) both factors that impact on the success of vaccination (Angelone et al., 2006). The Th2 bias in the APC response might be due to the fact that TGF- $\beta$ , progesterone and prostaglandin E2 produced by placental tissues increases Th2 cytokine production, as well as the presence of high levels of adenosine (an immunosuppressive endogenous purine metabolite) in neonatal plasma (Levy et al., 2006; Levy, 2007). Neonatal Tregs behave differently and are found in high concentrations in human foetal lymphoid tissues (Michaelsson et al., 2006) and cord blood (Godfrey et al., 2005; Wing et al., 2005; Flanagan et al., 2010). Neonatal Tregs may serve to control immunity to self antigens but can also limit adaptive responses by suppressing T cell proliferation and IFN- $\gamma$  production (Angelone et al., 2006). This suppression can be advantageous in protecting the neonates on first exposure to the outside environment, but can also be a disadvantage since it may limit responses to infections (Fernandez et al., 2008) and vaccines (Toka et al., 2004; Stober et al., 2005).

#### **1.5.2.2 Gamma-delta ( $\gamma\delta$ ) T cells**

These cell types develop mainly in the thymus and their TCR is formed through recombination events of the VDJ gene segments that encode antigen receptors, leading to repertoire diversity of the  $\gamma\delta$  TCR. Cells expressing the  $\gamma\delta$  TCR are CD3<sup>+</sup> T cells and

constitute 1-10% of the CD3<sup>+</sup> T cell population in peripheral blood, lymph nodes and spleen (Hayday, 2000; Grant et al., 2002). These cells do not express CD4 or CD8 and are therefore referred to double negative T cells. They do not recognize antigen bound to MHC-I or MHC-II molecules but rather recognize antigen in the context of the MHC class I related-protein CD1. CD1 presents glycolipid components of mycobacteria and other bacteria to a subset of  $\gamma\delta$  T cells (Grant et al., 2002); while another subset recognizes the MHC class I chain-related proteins known as MIC (Wu et al., 2002b).

Gamma delta T cells have both innate and adaptive features. For example non-peptide molecules from a variety of microorganisms can activate them. Human peripheral blood  $\gamma\delta$  T cells express effector functions via secretion of IFN- $\gamma$ , TNF- $\alpha$  and granzymes, and a subset of these cells that produce IL-17 are enriched in the dermis and intestinal lamina propria (Reviewed in (Vantourout and Hayday, 2013)). Activated  $\gamma\delta$  T cell effector functions are therefore similar to  $\alpha\beta$  T cells (Beetz et al., 2008). These cells have the ability to lyse infected macrophages thereby containing the dissemination of the infectious pathogen (Oliaro et al., 2005) and can also kill many tumor cells (Kabelitz et al., 2004). Depending on the physiology of the disease, human activated  $\gamma\delta$  T cells can produce Th2 cytokines including IL-4, as has been demonstrated in allergic asthma patients (Spinozzi et al., 1995).

Most studies of  $\gamma\delta$  T cells in early life were done in human neonates. Neonatal  $\gamma\delta$  T cells are highly functional and seem to be conditioned *in utero*, which give the neonate the ability to mount a strong cell mediated immune response in the absence of a mature  $\alpha\beta$  T cell immunity (Gibbons et al., 2009). Humans are born with high levels of  $\gamma\delta$  T cells but these decline with age.

#### **1.5.2.3 CD8 T cell activation and differentiation**

Cytotoxic T cells that express the CD8 co-receptor and recognize peptide–MHC class I complexes have a key role in clearing intracellular pathogens such as viruses. For



example, infection with a virus causes naive CD8<sup>+</sup> T cells expressing the virus-specific T cell receptors (TCRs) to clonally expand and differentiate into effector CD8<sup>+</sup> T cells that control the primary infection. These effector cytotoxic T lymphocytes (CTLs) can destroy virally infected cells through the targeted secretion of perforin and granzymes from lytic granules (Kaech and Cui, 2012). Most of these activated CTLs die by apoptosis after performing their effector function. However, an effective immune response ensures production of a stable population of antigen-specific long-lived memory CD8<sup>+</sup> T cells that can respond rapidly to clear secondary infections (Joshi and Kaech, 2008; Parish and Kaech, 2009; Jenkins and Griffiths, 2010). Besides their cytolytic activity, CD8<sup>+</sup> T cells (Tc cells) also have a function in the control of activation and differentiation of CD4 T cells mediated via secreted cytokines and chemokines, or via cell-cell interaction. CD8 T cells therefore display the ability to alter the balance of Th1/Th2 responses *in vivo* (Holmes et al., 1997; Noble et al., 1998).

#### **1.5.2.4 Tc1 and Tc2 CD8<sup>+</sup> T cells**

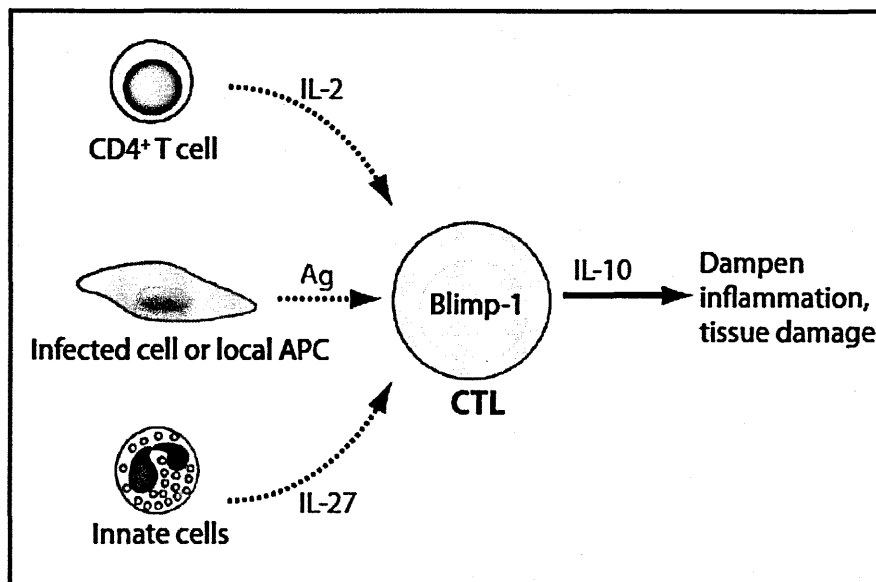
CD8<sup>+</sup> T cells are further classified into two distinct effector cell types, Tc1 and Tc2 cells, on the basis of their cytokine profiles. Tc1 cells characteristically produce type 1 cytokines IFN- $\gamma$ , IL-2 and TNF- $\alpha$  and Tc2 cells secrete type 2 cytokines IL-4, IL-5, IL-6, IL-10 and IL-13 thus mirroring the Th1 / Th2 dichotomy of CD4<sup>+</sup> T cells (Sad et al., 1995). The domination of a type 1 or type 2 cytokine response in the Tc cell populations will thus dictate the ability to defend against certain pathogens. Both type 1 and 2 cytokines secreted by activated CD8<sup>+</sup> T cells play important roles in regulating the overall immune response and the 2 arms antagonize one another (Xu et al., 2009) which will ultimately determine a beneficial or deleterious immune response (Seder, 1995).

IFN- $\gamma$  and IL-12 promote differentiation to Tc1 cells (Sad et al., 1995; Vukmanovic-Stejic et al., 2000). Tc1 cells elicit their effector function via the perforin pathway, as demonstrated in perforin-deficient mice which lack killing function. IL-12 induces expression of the transcription factor T-bet which is necessary for Tc1 differentiation (Sad

et al., 1995). A considerable amount of IL-4 is required for Tc2 differentiation, and inhibiting the action of IFN- $\gamma$  is crucial (Sad et al., 1995). Tc2 cells also possess the ability to kill via the perforin pathway, like their counterparts the Tc1 cells. The transcription factor GATA-3 is required for Tc2 differentiation and is induced by IL-4 (Sad et al., 1995). Several groups have reported the induction of functional Tc1 cells *in vivo* for example, lesions lepromatous leprosy lesions (Salgame et al., 1991) and HIV (Maggi et al., 1994),

#### **1.5.2.5 IL-10 Producing CD8+ T cells**

A regulatory role of effector CD8+ T cells in alleviating excessive tissue injury via the secretion of the immunosuppressive cytokine IL-10 has also been described (Sun et al., 2009; Palmer et al., 2010; Trandem et al., 2011). These effector CD8+ T cells have been studied in various viral infection models in the lung and brain and have been reported to produce large amounts of IL-10 at these local sites of infection, in contrast, CD4+ T cells are the main producers of IL-10 in secondary lymphoid organs (Sun et al., 2009; Palmer et al., 2010; Trandem et al., 2011). The IL-10+CD8+ population is thought to be a transient and reversible state of CD8+ T cell differentiation rather than a separate effector lineage, and helps to better balance pathogen clearance with bystander tissue damage. Following transfer of IL-10+ and IL-10- effector CD8+ T cells into a coronavirus-induced encephalitis model, they differentiate in similar frequencies into IL-10 producing populations (Trandem et al., 2011). The key roles of IL-2 and the transcriptional repressor (Blimp-1) have been demonstrated in an influenza virus infection model whereby IL-10 production was dependent on the synergistic cooperation between CD4+ T cell-derived IL-2 and innate (mainly neutrophil) derived IL-27. In the same model, the expression Blimp-1 by CD8+ T cells was required for IL-10 production (Sun and Lanier, 2011) (Figure 1. 6).



(Zhang and Bevan, 2011)

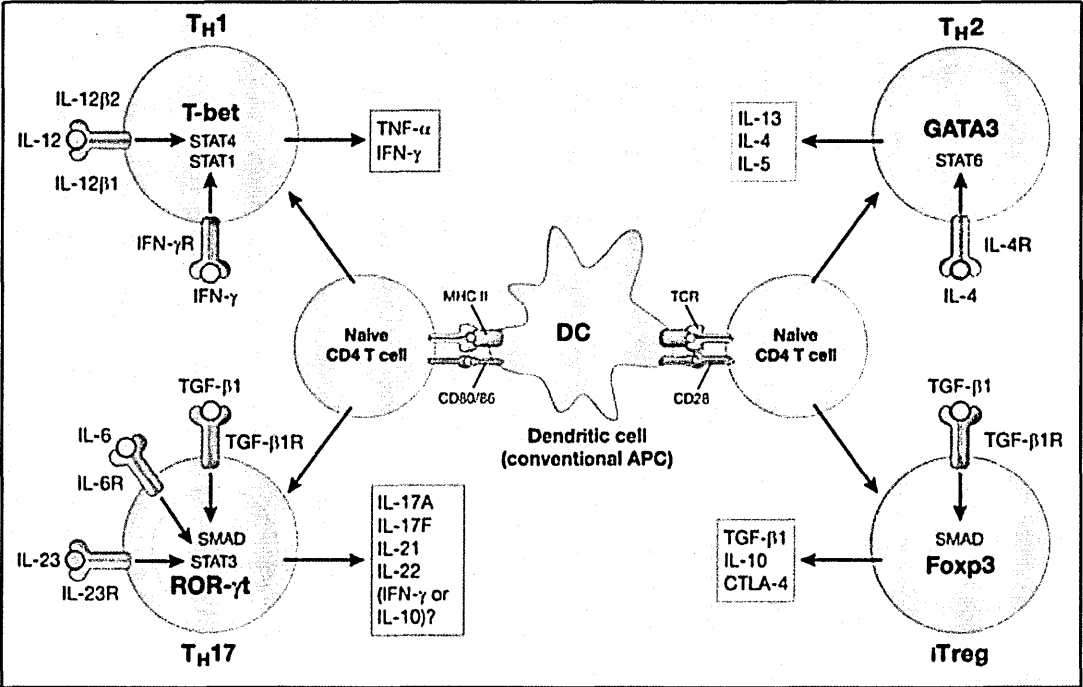
**Figure 1.6 Self-control by effector CD8+ T cells in infected peripheral tissues.** CD8+ T cells transiently produce IL-10 in a BLIMP-1 dependent manner after receiving additional local signals from CD4+ T cell-derived IL-2 and innate cell-derived IL-27. Production of IL-10 lead to control of local inflammation and tissue damage.

#### 1.5.2.6 CD4 T helper cell activation and differentiation

Naive CD4 T helper (Th) cells are activated when their T cell receptor (TCR) recognises a peptide bound in the groove of major histocompatibility complex (MHC) class II on the surface of antigen-presenting cells (APCs). This TCR and MHCII interaction (signal I) is required for CD4 T cell activation. The nature of the antigenic stimulus influences Th polarization (Constant and Bottomly, 1997; Tao et al., 1997). Studies using altered peptide ligands showed that the affinity between TCR and peptide-bound MHCII, that is the strength of TCR stimulation, affects lineage commitment (Sloan-Lancaster et al., 1997). Naive Th cells divide upon activation giving rise to effector T cells, each specific for the peptide–class II MHC complex by which it was activated (Goldsby et al., 2003; Kaiko et al., 2007). A second signal, co-stimulation (signal II) that is essential for efficient T cell activation is mediated through CD28-B7 and other surface molecules (Freeman et al., 1993; Schweitzer et al., 1997).

CD4+ T cells can be divided into 6 major subsets, designated Th1, Th2, Th9, Th17, Th22 and T regulatory cells (Mosmann et al., 2009). Differentiation of naive Th cells into one of

these subsets occurs within a few days of direct contact with APCs (Lafaille, 1998), the type produced being depending on many factors, in particular the antigen type and the local cytokine milieu. The different cell types can be distinguished by their unique cytokine production profiles and their functions, and also through the expression of different patterns of cell surface molecules as indicated in Table 1.2 below.



(Egwuagu, 2009)

**Figure 1.7 T-helper-cell differentiation.**At the initiation of CD4+ T cell activation, the antigen presenting dendritic cells secrete a variety of cytokines that instruct the naive T cell to activate one of several alternative T helper cell developmental pathways leading to Th1, Th2, Th17 or Treg lineage. Each T helper phenotype produces its signature cytokines that mediate its distinct immune functions.

**Table 1.2 CD4+ T cells subsets, their characteristics and functions**

| Subset | Tx factor | Effector molecules   | Homing receptors  | Target cells | Function           |
|--------|-----------|----------------------|-------------------|--------------|--------------------|
| Th1    | T-bet     | IFN-γ                | CXCR3, CCR5       | Macrophages  | Bacteria           |
| Th2    | DATA-3    | IL-4, IL-5, IL-13    | CCR4, CCR3, CRTh2 | Eosinophils  | Parasites          |
| Th17   | RORγt     | IL-17, IL-22, GM-CSF | CCR6+CCR4         | Neutrophils  | Bacteria and fungi |
| Th9    | PU.1      | IL-9                 | n.d               | Mast cells   | Helminthes         |
| Th22   | AHR       | IL-22                | CCR4+CCR10        | Epithelia    | Skin protection?   |
| TFH    | Bcl-6     | IL-21                | CXCR5             | B cells      | Antibody responses |
| Treg   | FoxP3     | TGF-β                | CCR7, CCR6, CCR4  | DC/T cells   | Regulation         |
| Tr1    | n.d       | IL-10                | CX3CR1            | T cells      | Regulation         |

n.d., not determined.

Tr1 = T cells with regulatory functions. Adapted from (Zielinski et al., 2011)

### 1.5.2.7 Th1 CD4+ T cells

The signature cytokine for Th1 cells is interferon (IFN)- $\gamma$ , although they can also produce other pro-inflammatory cytokines such as IL-2 and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) (Wan and Flavell, 2009; (Zhu et al., 2010a). Th1 cells are required for pro-inflammatory responses, migration of effector cells and cytolytic activity by CD8+ T cells (Reviewed in (Boyman and Sprent, 2012)). Production of IgG2 antibody by B cells is enhanced by Th1 cells in humans. IFN- $\gamma$  from these cells has an important role in the control of viral, bacterial, fungal, mycobacterial and parasitic infections (Dorman et al., 1999; Sen, 2001). Apart from pathogen elimination, the proinflammatory cytokines produced by Th1 cells can cause tissue damage, promote inflammatory disease (e.g. inflammatory bowel disease) and self-reactivity to antigens (Boyman and Sprent, 2012).

CD4+ T cells in secondary lymphoid organs are the main producers of IL-2. IL-2 has autocrine and paracrine effects. It has long been established that CD8+ T cell responses require CD4+ T cell help. This help is provided by CD4+ T cells or DCs via their IL-2 paracrine signaling, but it has recently been shown that antigen-specific CD8+ T cells rely more on autocrine signals from the IL-2 that they produce rather than that produced by CD4+ T cells (Feau et al., 2011). Differences in the types of cytokines produced by individual Th1 CD4 T cells have implications on their capacity to mediate effector functions or become sustained as memory cells or both. In this regard, CD4+ T cells that secrete only IFN- $\gamma$  have a limited capacity to develop into memory cells compared with IL-2 or IL-2 and IFN- $\gamma$  producing CD4 T cells (Hayashi et al., 2002; Wu et al., 2002a; Younes et al., 2003). This implies that, for example, vaccines eliciting a high frequency of single-positive IFN- $\gamma$  producing cells may be limited in their ability to provide durable protection.

An adequate CD4 Th1 response is critical to protect hosts from intracellular bacteria such as *Mycobacterium tuberculosis*, *Mycobacterium avium* (Kobayashi et al., 1997), *Salmonella typhimurium* (Mastroeni et al., 1999) and *Listeria monocytogenes* (Buchmeier and Schreiber, 1985); and also viruses such as herpes simplex virus (HSV) (Fujioka et al.,

1999), influenza A virus (Sareneva et al., 1998) and vaccinia virus (Tanaka-Kataoka et al., 1999). In addition to the beneficial effects of clearing foreign pathogens, the Th1 CD4 response also plays a role in tumour immunity (Micallef et al., 1997).

#### **1.5.2.8 Th2 CD4+ T cells**

Th2 signature cytokines include IL-4, IL-5, IL-13 (Zhu et al., 2010a) and IL-10 (Wan and Flavell, 2009). In addition, they also produce IL-9 and IL-25 (IL-17E) (Paul and Zhu, 2010). These cells were initially thought to be unable to produce IL-2, but results from later studies indicated that Th2 cells could often produce modest amounts of IL-2 (Zhu et al., 2010a). Th2 cells promote IgG1 and IgE class-switching and eosinophil recruitment (Arthur and Mason, 1986; Coffman and Carty, 1986; Mosmann et al., 1986) through the production of IL-4. IgE forms immune complexes by cross-linking the high affinity Fc receptors on the surface of cells of the innate immune system such as basophils and mast cells leading to their degranulation and Th2-type effector functions. These granules contain cytokines, chemokines, histamine, heparin, serotonin and proteases, the release of which cause smooth muscle constriction, increased vascular permeability and inflammatory cell recruitment (Paul and Zhu, 2010). Unlike the Th1 response, the Th2 response is often associated with robust antibody responses during which high levels of pathogen-specific immunoglobulin are generated to neutralize foreign organisms.

The Th2 response is key to resisting extracellular forms of pathogens, such as helminths and nematodes (Finkelman et al., 1991; Sher and Coffman, 1992). Th2 cells also play a vital role in mucosal immunity (Lai and Rogers, 2010). Similar to Th1 responses, an over-exuberant Th2 response can lead to pathological changes in the host. Chronic inflammatory airway diseases, such as atopic asthma and allergy, characterized by local infiltration with allergen-specific CD4 T cells and eosinophils, are attributed to over-exuberant Th2 immunity (Wierenga et al., 1990; Kay, 1991; Parronchi et al., 1991).

#### 1.5.2.9 Th17 CD4<sup>+</sup> T cells

Th17 cells are an effector subset of CD4<sup>+</sup> T cells (Boyton and Altmann, 2002). Upon activation, Th17 cells produce IL-17A, IL-17F, and IL-22, and have important functions in the clearance of extracellular bacteria and fungi, predominantly at mucosal surfaces. Retinoid-related orphan receptor (ROR) $\gamma$ t is a transcription factor induced by TGF- $\beta$  that is required for Th17 cell differentiation. ROR $\gamma$ t is not entirely induced by TGF- $\beta$  alone, but in conjunction with the proinflammatory cytokines IL-6, IL-21 and IL-23, which are all essential for activation of phosphorylation of the transcription factor STAT3 (Chen et al., 2007). Naive T cells in the presence of TGF- $\beta$  and other proinflammatory cytokines can differentiate into Th17 cells (Veldhoen et al., 2006). By contrast, naïve T cells in the presence of TGF- $\beta$  and absence of IL-6, they differentiate into induced Tregs (iTregs) (Bettelli et al., 2006). This would suggest that both cell types (Th17 and Tregs) can originate from the same precursor T cell which is dependent on the cytokine milieu (Bettelli et al., 2006).

A number of other cell types also produce similar cytokines to Th17 cells, namely  $\gamma\delta$  T cells, NK cells, NKT cells, and lymphoid tissue inducer (LTi) cells (Reviewed in (Gaffen, 2009)). Th17 cells function mainly at mucosal surfaces and can initiate proinflammatory danger signals that lead to enhanced neutrophil migration and production of antimicrobial proteins. Th17 cells also promote inflammatory pathology in many autoimmune diseases. Th17 cells have been implicated in vaccine-induced immunity and immunotherapy (Reviewed in (Khader et al., 2009)). In a study by Higgins *et al*, IL-17 contributed to vaccine-induced protective immunity against *B. pertussis*. In their study, administration of whole-cell pertussis vaccine resulted in induction of Th17 cells and when they neutralized IL-17, they observed a reduction in protection after pulmonary challenge with *B. pertussis* (Higgins et al., 2006a). Immunization of mice with whole cell pertussis (wP) vaccine induced Th1 and Th17 responses, but further studies in IFN- $\gamma$  deficient mice revealed a dominant role of Th1 cells in mediating vaccine induced protection (Ross et al., 2013). In contrast, the same study revealed that immunization of mice with acellular pertussis (aP)

vaccine resulted in the induction of Th2 and Th17 cells but not Th1 cells; this induction of Th17 responses to aP has not yet been fully studied in humans (Ross et al., 2013). Banus S *et al.* reported similar findings in mice infected with *B. pertussis* with wP stimulating a mixed Th1/Th17 cytokine profile but this was not the case with aP vaccine (Banus et al., 2008). Therefore, IL-17 appears to be a major modulator of the memory response generating strong protection in response to wP immunization, which is not observed in aP immunization. Incorporation of IL-17 in aP vaccines to improve efficacy by skewing the response to a dominant Th1/Th17 mixed profile is currently an attractive option (Poland, 2012).

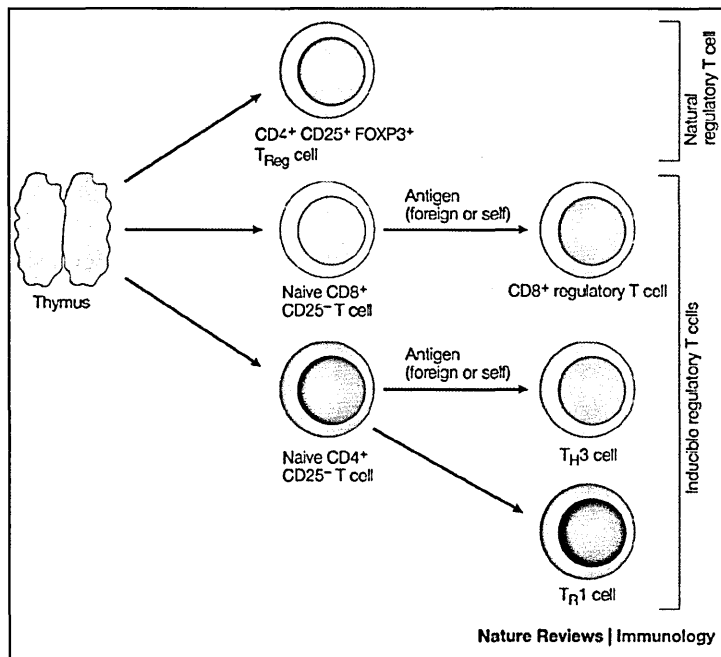
#### **1.5.2.10 CD4<sup>+</sup> regulatory T cells (Tregs)**

The primary role of Tregs is the modulation of immune responses. Tregs are broadly classified into two groups; natural Tregs (nTregs) and induced or adaptive Tregs (iTregs). nTregs develop in the thymus and are characterized by the expression of CD4 and CD25 molecules on their surface and by expression of forkhead box transcription factor P3 (FOXP3) in the nucleus that is crucial for their development (Sakaguchi S et al. 2006). They produce the cytokines TGF- $\beta$  and IL-10, which switch off or inhibit pro-inflammatory immune responses. This mechanism counteracts the inflammatory component of an immune response and thus avoids harm to the host (Sakaguchi, 2006; Kaufmann and Kabelitz, 2010).

There are currently two known main pathways for the generation of Tregs. The majority of Tregs are generated in the thymus during T cell maturation upon high avidity recognition of self-antigens and express FOXP3, called thymus-derived Tregs (also called natural Tregs) (Bluestone and Abbas, 2003). These cells express cell surface CD25 (IL-2R $\alpha$ ) and co-inhibitory receptor cytotoxic T lymphocyte antigen 4 (CTLA4) on their surface as well as in the cytoplasm (Sakaguchi et al., 2008).



The second pathway involves generation of Tregs in the periphery, upon encounter of a mature naïve CD4<sup>+</sup>T cell with persistent self-antigen. These peripherally derived Treg cells have also been called adaptive or induced Treg (iTreg)) cells (Sakaguchi et al., 2008). There is a subset of iTreg that are FOXP3<sup>+</sup> and indistinguishable from nTregs. The CD8 Tregs and Tr1 and Th3 are all FOXP3 negative. The iTreg cells are of three main types, namely, CD8<sup>+</sup> regulatory T cells from activated naïve CD8<sup>+</sup>CD25<sup>-</sup> T cells; and Th3 and Tr1 cells differentiated from activated naïve CD4<sup>+</sup>CD25<sup>-</sup> T cells (Mills, 2004). T regulatory 1 cells produce high amounts of IL-10 with no or low levels of IFN- $\gamma$  and no IL-4; on the other hand T helper 3 cells (Th3) secrete high amounts of TGF- $\beta$  (Figure 1.8) (Mills, 2004). CD8 T are regarded as cytotoxic cells that produce IFN- $\gamma$ , but these cells or a subtype of them have recently been described as IL-10 producers hence the name regulatory CD8<sup>+</sup> T cells (Figure 1.8) (Mills, 2004). A study in mice by Gratz and colleagues have revealed the life history of both thymic and peripherally derived Tregs and that they have similar steps of differentiation from naïve to effector to memory (Gratz et al., 2013).

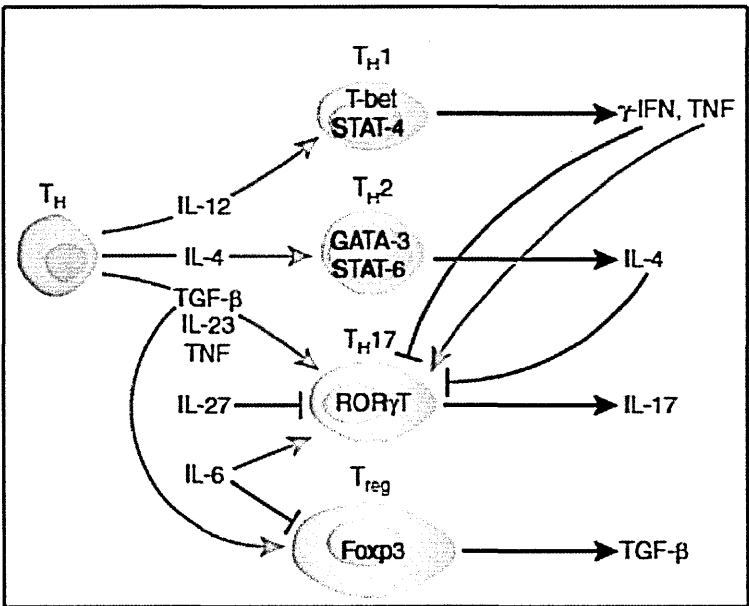


Mills K.H.G. 2004: (4) 841-855

**Figure 1.8 Differentiation of Treg cells.** Natural regulatory T cells express the cell-surface marker CD25 and the transcriptional repressor FOXP3 (forkhead box P3). Other populations of antigen-specific regulatory T cells can be induced from naïve CD4<sup>+</sup>CD25<sup>-</sup> or CD8<sup>+</sup>CD25<sup>-</sup> T cells in the periphery under the influence of semi-mature dendritic cells, interleukin-10 (IL-10), transforming growth factor- $\beta$  (TGF- $\beta$ ) and possibly interferon- $\alpha$  (IFN- $\alpha$ ).

**1.5.2.11 Transcription factors are critical regulators of Th cell differentiation**

Transcription factors are critical for CD4 T cell differentiation and cytokine production. At least two types of transcription factors are required for cell fate determination in each lineage, the master regulators and the signal transducer and activator of transcription (STAT) proteins (Figure 1.9).



**Figure 1.9 T-helper cell differentiation and regulation.** Green arrows indicate up-regulation, while red lines indicate inhibition. Transcription factors for particular lineages are placed in the nucleus (Steinman, 2007).

Some STAT proteins mediate induction of the expression of master regulators. The STATs and master regulators often work synergistically in regulating cytokine production by directly acting on cytokine genes. The essential transcription factors of Th lineages are T-bet/STAT4 (Th1), GATA-3/STAT5 (Th2), ROR $\gamma$ t/STAT3 (Th17), and FOXP3/STAT5 (iTreg) (Zhu and Paul, 2010).

The activation, differentiation, and expansion of Th cells are regulated by the activity and relative expression of specific transcription factors. In an ideal situation, the immune response should be sufficient to clear pathogens but controlled to prevent collateral damage. An inappropriate regulation may lead to chronic infection, whereas an uncontrolled response can lead to extensive self-tissue damage. An undesirable activation

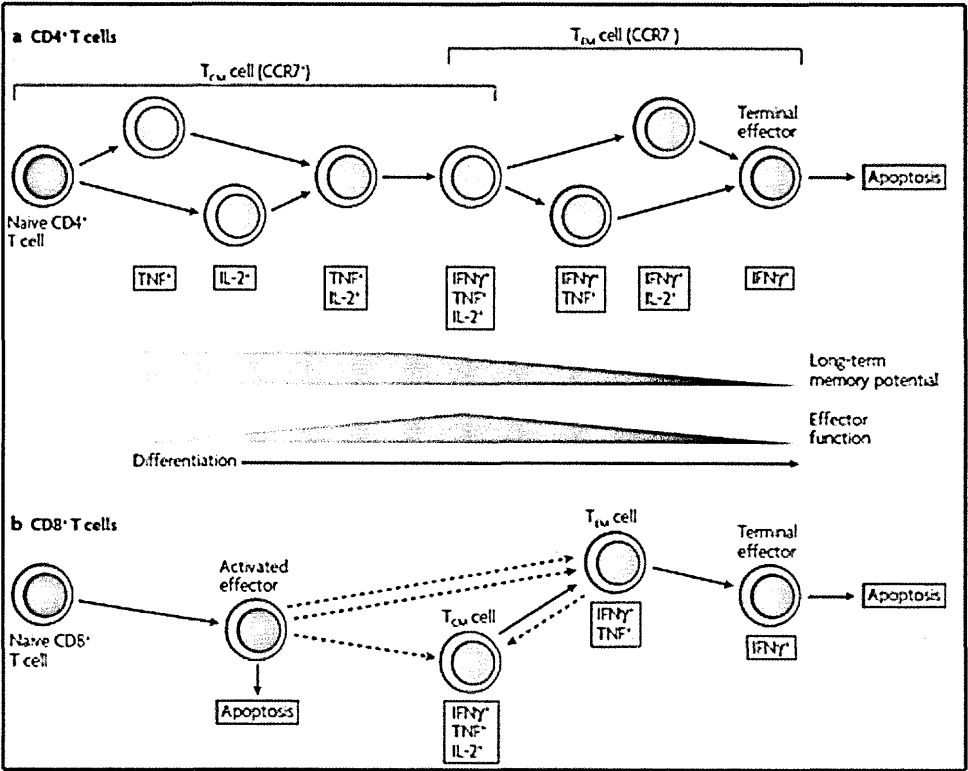
of Th1, Th17, or Th2 cells can also result in organ-specific autoimmune diseases (Th1, Th17) or allergic inflammatory diseases and asthma (Th2). Therefore, understanding the networks of cytokines and transcription factors produced during CD4<sup>+</sup> T-cell differentiation is critical for diagnosis and treatment of many immune-related human diseases (Zhu and Paul, 2010).

#### **1.5.2.12 Memory T cell responses after vaccination**

The ultimate goal of a vaccine is to develop long-lived immunological protection, whereby previous encounter with a pathogen is 'remembered', and either completely prevents re-infection or reduces the severity of the disease. This is called immunological memory.

Memory responses result from the clonal expansion and differentiation of antigen-specific lymphocytes that may persist for a lifetime. It is suggested that immediate protective memory is mediated by effector memory cells ( $T_{EM}$ ) that migrate to inflamed peripheral tissues and display immediate effector function (Bautista-Lopez et al., 2000; Lanzavecchia and Sallusto, 2000). By contrast, longer-term recall responses are thought to be mediated by central memory T cells ( $T_{CM}$ ) that reside in lymph nodes (LN) and circulate at low frequencies in the peripheral circulation. These  $T_{CM}$  have little or no effector function, but readily proliferate and differentiate to effector T cells upon antigenic stimulation (Campbell et al., 1998; Potech et al., 1999; Romero et al., 2007). Human  $T_{CM}$  are CD45RO<sup>+</sup> memory cells that constitutively express cell surface CCR7 and CD62L. These two receptors are also expressed by naive T cells and are required for entry through high endothelial venules and migration to T cell areas of secondary lymphoid organs (Campbell et al., 1998; Forster et al., 1999; Romero et al., 2007).  $T_{CM}$  have higher sensitivity to antigenic stimulation than naïve T cells, are less dependent on co-stimulation, and up-regulate CD40L to a greater extent, hence providing more effective stimulatory feedback to dendritic cells and B cells. Upon TCR stimulation,  $T_{CM}$  produce mainly IL-2, but efficiently differentiate to effector cells after proliferation to produce large amounts of effector cytokines (Figure 1.10) (Sallusto et al., 2004; Romero et al., 2007).

Human  $T_{EM}$  are memory cells that have lost the constitutive expression of CCR7, are heterogeneous for CD62L expression (Tripp et al., 1995; Sallusto et al., 2004; Romero et al., 2007), and display chemokine receptors and adhesion molecules that are required for homing to inflamed tissues (Figure 1.10) (Potsch et al., 1999; Xie et al., 1999; Romero et al., 2007). In contrast to  $T_{CM}$ ,  $T_{EM}$  are characterized by rapid effector function. Some  $T_{EM}$  lose their surface CD45RO and regain CD45RA expression. These have been called  $T_{EMRA}$  cells, and have been shown to have significant effector function, for example CD8  $T_{EMRA}$  carry high amounts of intracellular perforin (Baars et al., 2000; Sallusto et al., 2004).



**Figure 1.10 Models for effector and memory T cell differentiation.**

(a) CD4<sup>+</sup> T cell differentiation can be modeled as a linear process, in which cells progressively gain functionality with further differentiation, until they reach the stage that is optimized for their effector function (such as the production of interleukin2 (IL2), interferon- $\gamma$  (IFN- $\gamma$ ) and tumour-necrosis factor- $\alpha$  (TNF- $\alpha$ )). (b) Following stimulation, naive CD8<sup>+</sup> T cells fully differentiate into activated effector CD8<sup>+</sup> T cells that secrete IFN- $\gamma$ , most with cytolytic activity. Two models for the generation of CD8<sup>+</sup> central memory T cells ( $T_{CM}$  cells) and CD8<sup>+</sup> effector memory T cells ( $T_{EM}$  cells) are depicted by colour ('linear differentiation' is shown with black dotted arrows; 'fixed lineage' with red dotted arrows; and black arrows depict transitions for both models). Adapted from Seder RA et al. *Nat Rev Immunol.* 2008; 8(4): 247-58.

Understanding the memory T cell response to vaccines is key to designing new and better vaccines and has been well studied in BCG vaccinated humans. In a tetanus toxoid (TT) model of re-immunization of TT-immune adults, Cellerai *et al.* have shown that there was high expression of IL-7Ra and Bcl-2 prior to TT re-immunization, resembling central memory T cells and with production of IL-2 but not IFN- $\gamma$  *ex-vivo*. However, 5 to 15 days post vaccination with TT, a large proportion of the CD4<sup>+</sup> T cells acquired the ability to produce IFN- $\gamma$ , began to proliferate and down-regulated IL-7RA and Bcl-2. In contrast to this, 60 days and more post vaccination, TT-specific CD4<sup>+</sup> T cells were more of the IL-7RA<sup>high</sup> and Bcl-2<sup>high</sup> phenotype (Cellerai *et al.*, 2007). Memory T cell responses in *B. pertussis*-vaccinated pre-adolescent children demonstrated memory T cells specific to *B. pertussis* many years after vaccination. The authors found IFN- $\gamma$  and TNF- $\alpha$  producing cells were detected in 65% and 53% of the children respectively. Majority of the antigen-specific cells were CD45RA<sup>+</sup>CCR7<sup>+</sup> T<sub>EM</sub> cells whether defined by proliferation or cytokine production. Furthermore, the proliferative capacity of wP vaccinated children was higher than in aP vaccinated children although a longer time had elapsed for the wP vaccination than aP. The number of children that had detectable cytokine response was greater in the wP group than in the aP group (Smits *et al.*, 2013).

## 1.6 Measles vaccine

Measles, also known as Rubeola, is an infection of the respiratory system caused by a virus, specifically a paramyxovirus of the genus Morbillivirus. Morbilliviruses, like other paramyxoviruses, are enveloped, single-stranded, negative-sense RNA viruses. The measles virus genome is made up of 8 proteins that include haemagglutinin (H) and fusion (F) proteins. Neutralizing antibodies provide lifelong protection against the H protein after infection (de Swart *et al.*, 2005). The measles virus normally grows in the cells that line the back of the throat and lungs. A high fever persists for 2 or 3 days after contracting the virus (Orenstein *et al.*, 2004). Coughing, sneezing, close personal contact or direct contact with infected nasal or throat secretions spreads the virus from person to person. Measles is still common in many developing countries – particularly in parts of Africa and

Asia, and is increasing in developed countries such as the UK due to reduction in vaccination of infants ((WHO), 2012b). More than 20 million people are infected with measles each year and it remains one of the leading causes of death among young children globally, despite the availability of a safe and effective vaccine. Measles vaccine was first introduced in the 1960s and has a successful and an impressive record of safety and effectiveness. From 2001 to 2011 more than one billion children aged 9 months to 14 years who live in high-risk countries were vaccinated against the disease. Global measles deaths have decreased by 74% from 535,300 in 2000 to 139,300 in 2010 ((WHO), 2012b). The fourth Millennium Development Goal (MDG 4) aims to reduce the under-five mortality rate by two-thirds between 1990 and 2015, and reductions in measles deaths will make a major contribution to this aim ((WHO), 2012b).

Measles vaccine contains live, attenuated measles virus. It is available as a single-antigen preparation and in combination formulations, such as measles-rubella (MR), measles-mumps-rubella (MMR), and measles-mumps rubella- varicella (MMRV) vaccines. Measles vaccine is given subcutaneously or intramuscularly as a single or combined antigen, in a dose of 0.5ml. A single dose of measles-containing vaccine administered in the second year of life induces immunity in about 95% of vaccinees (King et al., 1991), and approximately 95% of persons who fail to respond to the first dose respond to a second dose (Watson et al., 1996). In the developing world it is given at 9-12 months of age, although studies suggest that earlier dosing may be beneficial (Aaby et al., 2010).

### **1.6.1 The immune responses to measles vaccine**

Both humoral and cellular immunity are induced by measles vaccination. Two independent studies have identified neutralizing antibody levels above 120–200 mIU/ml as a correlate of protection from measles (Chen et al., 1990; Samb et al., 1995). However, other studies have demonstrated that some MV-vaccinated subjects with low neutralizing antibody titres have detectable MV-specific T lymphocytes in their peripheral blood (Ward et al., 1995; Bautista-Lopez et al., 2000). Significant protection from measles has also

been shown for MV-vaccinated subjects with undetectable MV-specific neutralizing antibodies as compared with non-vaccinated subjects (Samb et al., 1995).

Both clinical and experimental data have shown that the cellular immune response is very important in recovery from measles virus infection (UytdeHaag et al., 1994). A generalized immunosuppression of T cell responses is associated with measles vaccination (Smedman et al., 1994), which may be more pronounced in infants (Gans et al., 1998; Gans et al., 1999; Gans et al., 2001). However, measles immunization at 9 months of age has been shown to induce CTL responses similar to that found in children or adults with acute measles, therefore mature CTL can be generated by vaccination in the first year of life (Jaye et al., 1998).

Measles vaccine induces a Th1 immune response, characterized by high levels of IFN- $\gamma$  and IL-2, quickly followed by a prolonged Th2 or mixed Th1/Th2 response with the production of IL-4, IL-13, and TNF- $\alpha$ , and persistently elevated IL-10. A delayed maturation of Th1 cell-mediated immunity was suggested in studies assessing responses to measles immunization at 6, 9 or 12 months of age, compared with antigen naive adult controls (Gans et al., 2001). This latter study aimed to investigate measles virus-specific cellular immunity in relation to age and the presence of passive maternal antibodies at the time of immunization in large cohorts of infants who were vaccinated at 6, 9, or 12 months. The authors found no age related differences in the three groups in T cell proliferation; in contrast, antibody titres to measles and mumps were lower in 6-month-old infants with no detectable mAbs. In the same study, when 9-month-old infants were immunized in the absence of mAbs, their antibody response was similar to those of the 12-month-old infants. A >90% protection from measles or at least severe disease was reported for infants aged 6 and 9 months by the use of both humoral and cellular immunity as a readout for vaccine immunogenicity (Gans et al., 2001). Other studies reported significantly lower antigen-specific T-cell proliferation and IL-12 responses to measles vaccination in infants and, although adult-like antigen-specific IFN- $\gamma$  responses were

observed, infant T cells showed a limited capacity to increase their IFN- $\gamma$  release in response to IL-12 supplementation, as compared with adults (Gans et al., 1999; Chougnnet et al., 2000).

Cellular responses cannot be over emphasized, but very little is known about the cellular immune responses that are induced by measles vaccination especially the relative importance of CD4+ and CD8+ T cells. Part of the reason for this is because measles virus induces immunosuppression and it has been difficult to induce stimulation in experiments. With new available technology like ELISPOT and multiparameter flow cytometry, this is becoming possible. Ovsyannikova and colleagues measured the frequency of measles virus-specific CD4+ and CD8+ memory T cells years after completion of a two-dose vaccine series in adults who were either seronegative or highly seropositive for measles vaccine IgG-specific antibody and reported similar predominance of IFN- $\gamma$  over IL-4 in both groups. The median frequency of measles virus-positive CD8+ T cells secreting IFN- $\gamma$  as measured an ELISPOT assay was 0.09% in seronegative subjects while in highly seropositive subjects, it was 0.43% in ( $P = 0.04$ ). With CD4+ T cells secreting IL-4 in response to stimulation with measles virus, the frequency was 0.03% in seronegative subjects and 0.09% in highly seropositive subjects ( $P = 0.005$ ). These results provide evidence that measles virus-specific cellular immunity can be detected post measles vaccination in humans (Ovsyannikova et al., 2003).

### **1.7 Diphtheria vaccine**

Diphtheria is a disease caused by infection with bacteria called *Corynebacteria diphtheriae*. Dissemination is usually by air droplets and symptoms begin 1 to 5 days after infection. The first sign is usually a mild sore throat and pain on swallowing. It is also usual to experience a low-grade fever, nausea, vomiting, headache and a fast heart rate. In most cases the bacteria produce a powerful toxin (toxigenic diphtheria) that can destroy nearby tissue and cause a membrane of dead cells to form. The toxin can also travel through the bloodstream to the heart and nerves; resulting in damage to the heart muscle



which can lead to heart failure and sudden death (Department of Health, 2003). Before the introduction of the antitoxin in the 1890s, case-fatality rates from some diphtheria outbreaks reached or exceeded 50%. Although most infections with *C. diphtheriae* are asymptomatic or run a relatively mild clinical course, high case-fatality rates (>10%) have been reported even in recent outbreaks ((WHO), 2006a). Indeed, the while number of cases has decreased, the fatality rate has not fallen despite modern management (Chin, 2000). The worldwide total number of reported cases ranged from 74,000–94,000 per year in the 1970s to 23,000–28,000 per year in 1990–1992, with a 70% global decline in diphtheria morbidity over this 20-year period.

Diphtheria vaccine is a preparation of diphtheria toxoid. Usually it is available as a preparation adsorbed with aluminium hydroxide or phosphate and often combined with other toxoids or vaccines. Currently, diphtheria toxoid is almost exclusively available in combination with tetanus toxoid (T) as DT/dT, or with tetanus and pertussis vaccine as DTP (the origin of the pertussis component either being whole-cell (wP) or acellular (aP)). Antibodies to the variable K-antigens of the bacterial cell wall induce type-specific protection against carriage and mild forms of localised disease. Immunity to severe forms of localised and systemic disease depends mainly on the presence of IgG antitoxin antibodies ((WHO), 2006a). Antibody concentrations of  $\geq 0.01$  IU/ml are thought to provide some protection, while levels of  $\geq 0.1$  IU/ml are considered fully protective and are associated with long-term protective immunity ((WHO), 2006a). Circulating antitoxin levels below 0.01 IU/ml are non-protective.

### **1.8 Tetanus vaccine**

Tetanus (commonly known as 'lockjaw') is caused by the bacterium *Clostridium tetani*. Unlike diphtheria and pertussis, tetanus is not communicable. Spores of the bacteria are present in soil and manure and can be acquired through a scratch, puncture wound, burn or more serious injury. The bacteria release toxins, which act locally at the site of an injury and also on the central nervous system (CNS) to cause the disease symptoms. The

majority of cases occur within 3 days to 3 weeks of exposure (Department of Health, 2003).

Neonatal tetanus was considered a major public health problem at the end of the 1980s. The WHO estimated that in the year 2000, 309,000 people died of tetanus ((WHO), 2001), including 200,000 cases of neonatal tetanus (World Health Organization (WHO), 2002), a reduction of about 75% when compared to 1988. Neonatal tetanus now contributes to about 5% of global neonatal mortality, compared to 14% in 1993 ((WHO), 1996a).

Tetanus toxoid vaccines are available as a single toxoid (TT), or adsorbed with aluminium phosphate or hydroxide, combined with diphtheria toxoid (DT) or low-dose diphtheria toxoid (dT), or in combination with diphtheria and pertussis vaccines (DTwP, DTaP, dTaP). WHO stipulates that not less than 60 IU should be administered per dose (Myers et al., 1982). DTwP is used mostly in developing countries because it is much cheaper to produce than aP, and is more immunogenic. However, it is associated with more side-effects than aP and as a result wP was replaced with aP in the developed world due to the better safety-profile of the latter ((WHO), 2009b), albeit at the expense of the inferior immunogenicity.

Tetanus toxoid is a T cell dependent antigen, which induces long-lasting immunity against tetanus (Kroon et al., 1999) via the production of anti-tetanus antibodies. Protection can be achieved through active (tetanus vaccination) or passive (tetanus-specific immunoglobulin) immunization. Immunization of pregnant women with at least two doses of tetanus toxoid-containing vaccine: the first dose as early as possible during pregnancy and the second dose at least 4 weeks later is recommended by WHO. The immunized mother passes antitoxin IgG Abs via the placenta to the foetus, thereby preventing neonatal tetanus ((WHO), 2006b). This has been incorporated into antenatal care programs in many African countries leading to a dramatic reduction in death from neonatal tetanus.

The immune response to TT vaccination has been extensively studied in populations in developed countries to show that protection is dependent on the amount of IgG antibodies, the subclass distribution, which is mainly dominated by IgG1, and the avidity of the produced antibodies (Kroon et al., 1999). To obtain long-lasting immunity booster doses are required approximately every 10 years throughout life. Past studies on TT vaccination in developing countries concentrated mostly on the effect that parasite infections had on the vaccine efficacy. To this effect, studies conducted on onchocerciasis infected patients reported low responses to TT vaccination as defined by reduced IFN- $\gamma$  production by T cells and reduced antibody concentrations (Prost et al., 1983; Cooper et al., 1998; Cooper et al., 1999). The cytokine balance in schistosomiasis patients after tetanus vaccination was skewed towards a Th2 response compared to a Th1 and Th0 response in uninfected controls (Sabin et al., 1996). These studies were done on young adults. In a study in Gabon in which TT booster was administered to school children between the ages of 7 and 12 years; semi-urban children showed a Th1 cytokine profile while a Th2 cytokine profile was observed in rural children (van Riet et al., 2008). Mayer *et al.* demonstrated that IFN- $\gamma$  production after TT booster vaccination of healthy donors was exclusively from CD4<sup>+</sup> T cells. In vitro assays in which they depleted CD8<sup>+</sup> T cells and NK cells did not affect IFN- $\gamma$  production; in contrast, upon depletion of CD4<sup>+</sup> T cells, IFN- $\gamma$  production was almost non existence (Meyers et al., 2002). However, responses to vaccines containing alum adjuvant such as tetanus toxoid are often Th2 biased (Petrovsky and Aguilar, 2004).

While most studies have reported that IFN- $\gamma$  is the predominant cytokine produced by TT-specific CD4<sup>+</sup> T cells in humans (Fernandez et al., 1994; Cellerai et al., 2007), more recently an adult study revealed that the memory responses for Th1, Th2, IL-10 and IL-17 cytokines could be identified following TT immunization, which shows that the response to TT has a heterogeneous cytokine profile (Livingston et al., 2013).

### 1.9 Whole-cell Pertussis vaccine (wP)

Pertussis is caused by infection with the bacterium *Bordetella pertussis*, a strictly human pathogen that causes the respiratory disease whooping cough. The bacteria are transmitted from infected to susceptible individuals through cough droplets. The incubation period is usually 9–10 days (range 6–20 days), after which the infection progresses to the catarrhal phase (including cough), then there is progression to the paroxysmal phase that can persist for months, followed by the convalescent stage (Heininger et al., 1997). The catarrhal, paroxysmal and convalescent stages combined typically last for approximately 3 months ((WHO), 2010).

*Bordetella* species may change their phenotypic characteristics and show different expression of virulence factors depending upon environmental conditions. These factors include the nature of the pertussis toxin (PT), filamentous haemagglutinin (FHA), pertactin (PRN), fimbriae (FIM) type 2 and type 3, adenylate cyclase toxin (ACT), tracheal cytotoxin (TCT), lipooligosaccharide and *B. pertussis* endotoxin ((WHO), 2010). Pertussis pathogenesis is not completely understood but FHA, PRN and FIM facilitate attachment to targeted host cells, and PT, TCT and ACT enable the bacterium to destroy the epithelial lining and evade the host's immune system ((WHO), 2010).

Worldwide, pertussis remains an important cause of infant death and therefore is a public health concern even in countries with high vaccination coverage. WHO estimates suggest that about 16 million cases of pertussis occurred worldwide in 2008, 95% of which were in developing countries, and that about 195,000 children died from the disease (Black et al., 2010). The incidence of pertussis is on the rise despite the availability of an effective vaccine that is used worldwide, in part due to decreased vaccine uptake in the developed world. The number of cases of pertussis was almost zero in the 1980s in California; this has increased in 2010 to match rates reported in 1947 (Kuehn, 2010). The epidemiology of pertussis is also changing and the infection is increasingly being found in older children

and adolescents. This was demonstrated in a serological study in the United States that found 21% of adults with a cough lasting greater than 2 weeks had pertussis (Wright et al., 1995). The UK has high vaccine coverage and a booster dose of pertussis is included in the preschool booster. This has led to a decline in the cases of pertussis between 1998 and 2009. In spite of this, UK has been having periodic outbreaks since 2011 particularly among adolescents and adults ((HPS), 2012), while the increase in cases in 2012 affected mainly very young infants ((HPS), 2012).

Pertussis vaccine has been highly successful in preventing severe pertussis in infants worldwide. Two types of pertussis vaccine are available: whole-cell (wP) vaccines based on killed *B. pertussis* organisms at a concentration of more than 4 IU, and acellular (aP) vaccines based on highly purified, selected components of the whole cell agent (Njamkepo et al., 2002). Most of the licensed aP vaccines contain 4 or 5 separately purified pertussis antigens (PT, FHA, PRN and FIM 2/3). The wP vaccines are based on standardized cultures of selected *B. pertussis* strains that are subsequently killed, usually by heating and treatment with formalin. Most wP vaccines are combined with diphtheria toxoid and tetanus toxoid. All available wP vaccines contain aluminium salts as an adjuvant ((WHO), 2010). Vaccine trials have demonstrated that antibody levels to *B. pertussis* antigens including PT, FHA, PRN and fimbriae correlate with protection against whooping cough. High anti-*B. pertussis* IgG was found in mice to correlate with rapid clearance of the bacteria in a respiratory infection model, but a pertussis-specific CMI response was required for complete elimination of the organism from the lungs. *In vitro*, *B. pertussis* survives in human monocytes and other mammalian cells, therefore, a CMI response may be necessary for eliminating this pathogen which has the potential of becoming an intracellular reservoir of bacteria (Gwendolyn and Richard, 1998). *B. pertussis* specific Th1 cells are found in humans after vaccination or infection, implying a role for cell-mediated immunity.

### **1.10 The diphtheria, tetanus, whole cell pertussis (DTwP) combined vaccine**

The DTwP vaccine is prepared from the inactivated diphtheria and tetanus toxins (rendered harmless and called toxoids) combined with one or more strains (types) of whole pertussis bacteria that have been killed. There is still no immune correlate of protection for pertussis and earlier studies relied on geometric mean titres (Miller et al., 1995).

Earlier studies of DTwP induced immunity in the UK reported the possible need for a booster dose of the vaccine in the second year of life, as was practiced in North America and many European countries. Despite booster doses in these countries, there were still reported cases of whooping cough in adults after many years of low incidence, indicating that the protection does not always extend into adulthood but can be boosted from natural exposure (Reviewed in (Miller et al., 1995). Miller and colleagues studied the responses of pre-school children given a booster dose of DTwP after a three dose primary immunization at 3, 5, and 10 months (former UK schedule) and they observed more adverse events in children administered with DTwP than the DT control group, but a good antibody response was obtained after the DTwP boost (Miller et al., 1995). Another group reported similar findings; a good boosting was obtained at 18 months to all components of DTwP vaccine, and it was well tolerated (Nolan et al., 1998). With the advent of DTaP, introduced in the 1970s, many studies were carried out in children to compare DTwP and DTaP vaccines reporting more adverse effects with DTwP compared to DTaP (Trollfors et al., 1995; Greco et al., 1996; Gustafsson et al., 1996; Miller et al., 1997; Simondon et al., 1997).

DTwP is generally thought to boost a Th2 profile with a reduced IFN- $\gamma$  to IL-4 ratio (Graham et al., 1993; Lindblad et al., 1997; Moran et al., 1999; Fischer et al., 2000). However, studies have shown that that vaccination with the wP component alone induces a Th1 (IFN- $\gamma$  and IL-2) cellular response (Ryan et al., 1997), while those vaccinated with

aP show a mixed Th1/Th2 cytokine profile (Ryan et al., 1998). Other studies in infants after immunization have reported a selective activation of Th1 cells with wP and a strong antibody and Th2 biased response with aP (Ausiello et al., 1997; Ryan et al., 1998; Dirix et al., 2009; White et al., 2010). Although the Th2/Th1 cytokine ratio was higher in aP than wP vaccinees, tetanus-specific T cells responses were higher in the latter group (Dirix et al., 2009). Similar findings were reported in murine respiratory challenge models of parapertussis (Sato et al., 1980) and revealed a requirement of both humoral and cell mediated immunity for the clearance of *B. pertussis* from mice lungs (Mills et al., 1998). Antigen-specific Th17 cells are induced by wP vaccination in immunized mice (Higgins et al., 2006a), which together with Th1 cells promote bacterial clearance via the recruitment and induction of macrophages and neutrophils thereby eliminating *B. pertussis* (Mahon and Mills, 1999; Higgins et al., 2006a). Thus Th1 cells and Th17 cells have a protective role in immunity against *B. pertussis* infection, and ideally should be induced by vaccination.

### **1.11 Vaccine adjuvants**

Glenny and colleagues first described the adjuvant effect of aluminium compounds (Glenny et al., 1926; Lindblad, 2004). Adjuvants may be molecules, compounds, or macromolecular complexes that have the ability to enhance the potency, quality, or longevity of specific immune responses to antigens. Adjuvants should not be too toxic. There are broadly two classes of adjuvants, immune modulators and delivery vehicles, but some adjuvants can exhibit both properties. Adjuvants target antigen-presenting cells (APC) which are central in both innate and adaptive immunity (Alving et al., 2012).

It has been suggested more recently, that the antigens adsorbed onto the aluminium salts are presented in a particulate multivalent form, making them more efficiently internalized by APCs (Morefield et al., 2005). Many of the EPI vaccines such as DTwP and Hepatitis B vaccine contain aluminium-based adjuvants (Clements and Griffiths, 2002), in order to enhance their immunogenicity. However, aluminium salts primarily enhance Th2-driven

antibody responses and have little effect on Th1-type responses, which are instrumental for protection against many pathogens (Leroux-Roels, 2010).

A number of toll-like receptor (TLR) agonists have recently been discovered as potent vaccine adjuvants. TLRs are pathogen recognition receptors that recognize pathogen associated molecular patterns. These TLRs are immunoenhancers that function via direct stimulation of cells of the innate immune system such as monocytes, macrophages and dendritic cells (Skeiky et al., 2004; Didierlaurent et al., 2009; Coffman et al., 2010). There are 10 known TLR agonists recognized by humans. TLR2, 3, 4, 5, 7, 8, and 9 are the best characterized molecules which are derived from bacterial or viral components, all of which have reached clinical testing as vaccine adjuvants (Barton, 2007; Bode et al., 2011; Tomai and Vasilakos, 2011). In the mouse, there are 12 recognized TLR members (Kumar et al., 2013; Reynolds and Dong, 2013). Human TLR2 is engaged by bacterial lipopeptides (Philbin and Levy, 2009). TLR3 agonists are endosomal receptors derived from viral double stranded RNA such as poly (I:C), which has been used in clinical trials for cancer (Seya et al., 2003). TLR4 is a plasma membrane receptor found on human dendritic cells and macrophages, which bind bacterial lipopolysaccharide, monophosphoryl lipid A (MPLA). Bacterial flagellin is a TLR5 agonist, single stranded RNA is a TLR7/8 agonist, and CpG oligonucleotides act as TLR9 agonists. TLR7/8 agonists hold promise as a novel neonatal vaccine adjuvant as it stimulates a strong Th1 response in neonates (Philbin and Levy, 2007; Burl et al., 2011). TLR8 is also expressed by human Treg cells and plays a key role in the reversal of Treg function when exposed to TLR8 agonists, thus overcoming immunosuppression and enhancing immunity (Philbin and Levy, 2007; Burl et al., 2011).

### **1.12 Immune polarization and vaccines**

The classic assumption is that live vaccines such as BCG and measles vaccine induce type 1 immunity, whereas the killed / inactivated vaccines such as DTwP or HBV skew towards type 2 responses. While this might be true in animals, the situation is more complex in humans. The measles vaccine response is often type 2 skewed in human



infants (Karp et al., 1996), and the pertussis component of DTwP can induce type 1 pro-inflammatory responses (Sharma and Pichichero, 2012). Furthermore, aside from the classical Th1/Th2 dichotomy, other arms of the immune system need to be considered such as the induction of Th17 cells and Tregs following vaccination. Th17 CD4 T cells are a recently described lineage, that confer protection against extracellular bacteria and fungi, particularly at epithelial surfaces (Weaver et al., 2006; Bettelli et al., 2007; Weaver and Murphy, 2007). IL-17 producing Th17 cells are primed upon administering whole cell pertussis vaccine, and when IL-17 is neutralized there is a significant reduction in vaccine-conferred protection in mice (Higgins et al., 2006b).

### **1.13 Non-specific or heterologous effects of vaccines**

Aside from inducing vaccine specific memory responses, there is compelling evidence that vaccines also have non-specific effects on morbidity and mortality from non-vaccine infections. These morbidity / mortality effects therefore cannot be accounted for by decreased cases of the targeted vaccine disease. These so called non-specific effects (NSE) or heterologous effects of vaccines have been overlooked in the past because it was assumed that the impact on survival would be proportional to protection against the targeted infection. Observational studies have repeatedly shown that vaccines can alter all cause mortality, and now randomized trials are emerging which confirm these findings (Knudsen et al., 1996; Aaby et al., 2003a).

One of the first widely accepted evidence that vaccines can alter all cause mortality came from the high-titre measles vaccine (HTMV) trials. In 1989 HTMV was recommended by WHO for use in high measles endemic settings. Randomized trials conducted in Senegal, Guinea Bissau and Haiti showed that although HTMV protects against measles infection, there was increased female mortality in the HTMV females compared to those that received the standard MV and compared to males in both groups (Knudsen et al., 1996; Aaby et al., 2003a). As a result HTMV was withdrawn by WHO in 1992. Later it was

suggested that it was the subsequent DTwP dose that increased the mortality two-fold in females (Aaby et al., 2003c).

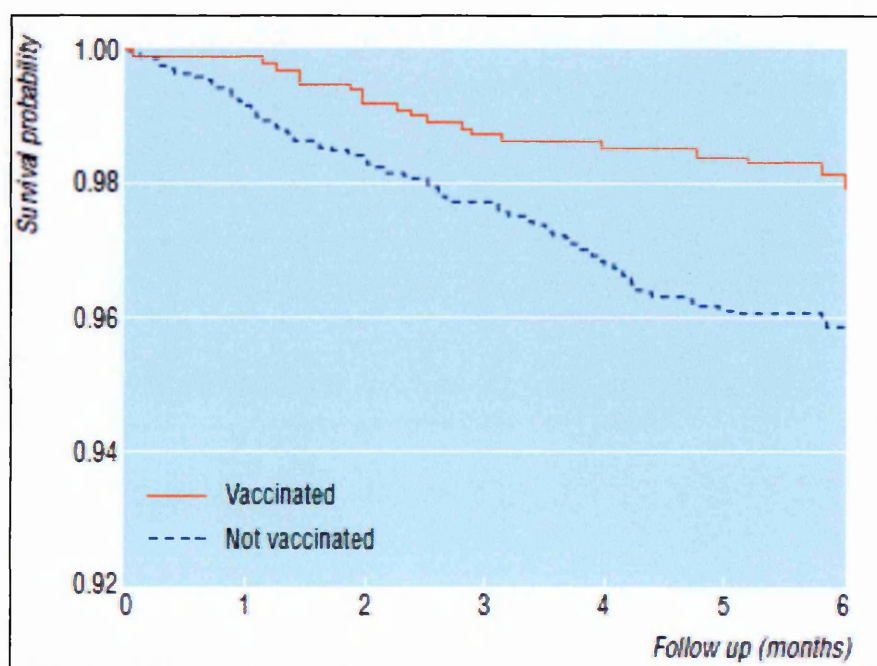
Bacillus Calmette-Guerin vaccine (BCG) has been shown in randomized trials to reduce mortality from infections other than tuberculosis (Roth et al., 2006; Roth et al., 2010; Shann, 2010; Aaby and Benn, 2011) suggesting a non-specific protective effect. The fact that BCG is used as a treatment for bladder cancer further confirms that it can have non-specific immune effects unrelated to induction of immunity against TB (Alexandroff et al., 1999). These non-specific effects are maximal in the first six months after vaccination and are largely determined by the last vaccine administered (Shann, 2010). In general, live vaccines such as measles vaccine (MV) and BCG have been shown to have non-specific beneficial effects. Unfortunately inactivated vaccines, including DTwP, have been shown to have non-specific deleterious effects, with females being more susceptible than males (Aaby et al., 2004b; Veirum et al., 2005; Aaby et al., 2006a). These observations have been highly controversial and as yet the mechanisms have not been elucidated although a number of potential mechanisms have been postulated (Flanagan et al., 2011; Flanagan et al., 2013b).

#### **1.13.1 Non-specific effects of Measles Vaccine**

Early observational studies suggested that measles vaccination could reduce all-cause mortality, and that the effect could not be attributed solely to a reduction in measles infections (Aaby et al., 1996b; Aaby et al., 1996a; Aaby et al., 2003a). Many studies in Africa reported a 40% or more reduction in child mortality after the introduction of measles vaccine in the late 1970s and early 1980s (Aaby et al., 1981; Aaby et al., 1984; Aaby et al., 1989; Aaby et al., 1993; du Loû et al., 1995). Studies in other countries reporting similar findings included Bangladesh and Haiti (Clemens et al., 1990; Holt et al., 1990). This reduction was larger than was expected as measles infection in these regions causes considerably less than 40% of all-cause mortality, leading to the suggestion that measles vaccine may have non-specific beneficial effects that is protecting against infections other

than measles (Aaby et al., 1995). A follow-up study by Kristensen *et al.*, in which the authors assessed the association between routine childhood vaccination and survival in Guinea Bissau, the mortality ratio of measles vaccinated children was 0.48 (0.27 to 0.87). However, on the exclusion of measles deaths, the mortality ration was 0.51 (0.28 to 0.95) indicating a beneficial protective effect against dying from the measles vaccine (Figure 1.12) (Kristensen, 2000).

More recently randomized trials in Sudan and Guinea-Bissau of providing an additional early dose of MV have confirmed this effect of reducing death from non-measles causes (Aaby et al., 2006a; Aaby et al., 2010). One of these studies was a large 2 dose RCT in which MV was administered at 4.5 months of age and the second dose at 9 months of age. The results revealed a 30% (6 – 48%) reduction in mortality between 4.5 and 36 months compared with those that received a single dose of standard MV at 9 months (Aaby et al., 2010). This effect could not be accounted for by a reduction in measles deaths alone, and remained at 24% (0 – 45) after the contribution of measles deaths was allowed for.



**Figure 1.11 Kaplan-Meier survival curves for recipients and non-recipients of measles vaccine.** Six months follow up of 3,414 children initially aged 7-13 months, Guinea Bissau, 1990-6 (Kristensen, 2000).

### **1.13.2 Non-specific effects of DTwP Vaccine**

When DTwP vaccination was introduced into Guinea-Bissau there was a significant increase in mortality in those children that received the vaccine (Aaby et al., 2004a). Thus, those children in Guinea-Bissau aged 2-8 months that were administered with DTwP had higher mortality than those that did not receive DTwP, the mortality rate ratio (MR) being 1.92 (95% CI: 1.04,3.52) for the vaccinated group. The reasons for not receiving the DTwP vaccine included illness or travelling, or occasionally because DTwP was not available (Aaby et al., 2004a). There was no evidence in this study to suggest that the unvaccinated children were a low risk group, indeed unvaccinated children are usually a high-risk group (Fine et al., 2009).

It has been deemed unethical by WHO to delay DTwP in randomized trials (Shann, 2010). However, a recent analysis of all available studies (n=35) of DTwP vaccination on child survival concluded the following: 1) DTwP vaccinated children had higher mortality than unvaccinated children; 2) DTwP vaccinated females have higher mortality than males in all studies; and 3) Reducing time of exposure to DTwP as most recent vaccine by giving BCG or MV reduced child mortality (Aaby et al., 2012).

### **1.14 The interaction between DTwP and measles vaccine**

In The Gambia EPI schedule, three doses of DTwP are given at 8, 12 and 16 weeks of age, and measles vaccine is given at 9 months of age. However, vaccines are frequently delayed, for reasons such as illness, inability to attend clinic, lack of electricity to store vaccines, particularly in resource poor settings. WHO recommend that when a child presents for routine vaccinations, any missed vaccines be given (Decker, 2001). Thus children who come for MV and are missing one or more DTwP vaccines are given both vaccines simultaneously. Several observational studies suggest that receiving DTP together with MV is associated with higher mortality than those receiving MV only. A Gambian study showed a relative risk of death of 5.59 (2.10; 14.8) for children having a third DTP (DTP3) together with MV compared with children having MV only (Aaby et al.,

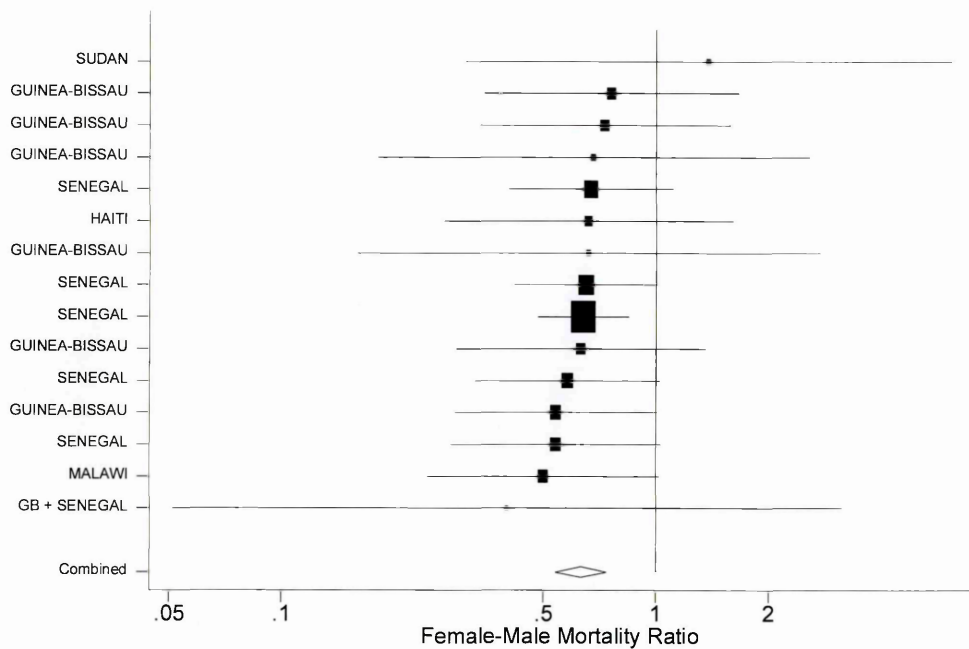
2006b). A study from Congo showed a mortality rate ratio (MRR) of 5.38 (1.37; 21.15) (Aaby et al., 2006a), and from Malawi of 5.27 (1.11; 25.0) for children who received DTwP together with MV compared with children who received MV only (Aaby et al., August 2006). Part of the explanation for these observations might be selection bias whereby children who come late for their vaccines have poorer health seeking behaviour. However, the effect estimates are so large that it seems implausible that they are due to selection bias alone (Agergaard et al., 2011).

### **1.15 Sex differences in non-specific effects of vaccination**

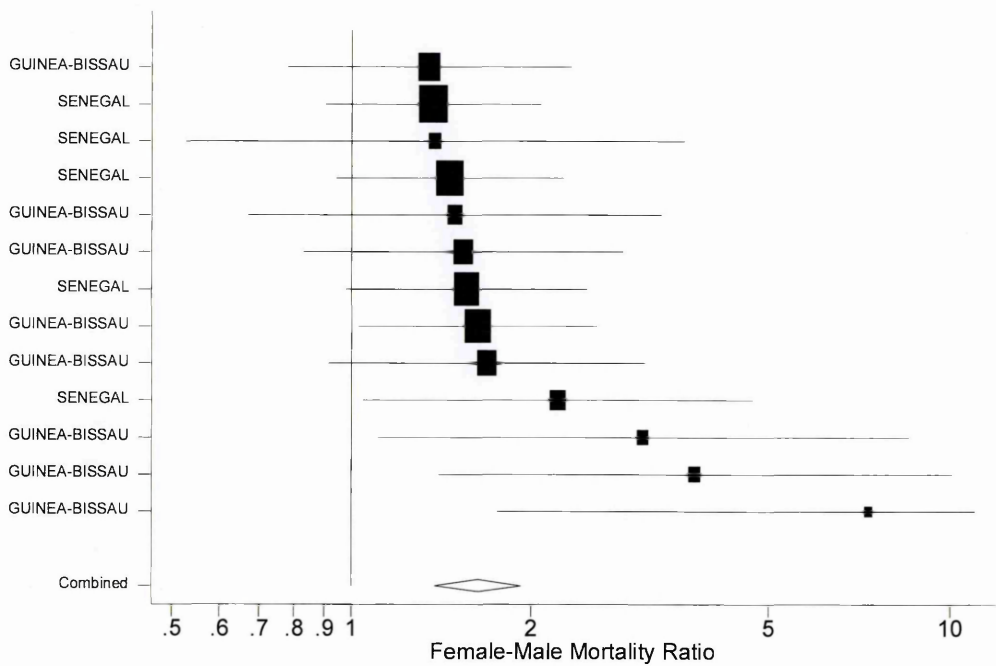
Generally it is females that are more susceptible to the non-specific / heterologous effects of vaccines than males. Thus females seem to benefit more than males following measles vaccination; but conversely females fare worse than males after DTwP vaccination (Figure 1.13a and 1.13b respectively) (Aaby et al., 2002; Aaby et al., 2004a; Aaby et al., 2004b; Veirum et al., 2005; Aaby et al., 2006b; Benn et al., 2009a). For example, overall female mortality is increased after DTwP vaccination but males remain unaffected (Kristensen, 2000; Aaby et al., 2004a; Jensen et al., 2007). By contrast, BCG and measles vaccination lower mortality in females, with a less significant effect in males (Aaby et al., 1995; Aaby et al., 2003b; Roth et al., 2006).

In a randomized study in Guinea Bissau, administration of DTP+OPV with MV had negative effects on growth and morbidity if girls (Agergaard et al., 2011). In the same study, whenever adverse events were observed in the group that received MV+DTP+OPV, the authors consistently found a female-male ratio below 1 in the MV+OPV group and above 1 in the MV+DTP+OPV group (Agergaard et al., 2011).

A



B



**Figure 1.12** The female / male mortality is consistently  $<1.0$  following measles vaccination (A), whereas it is  $>1.0$  after DTwP vaccination (B) in trials conducted in Africa and Haiti. This suggests that females benefit more from the NSE of MV, and fare worse than males after DTwP. (P Aaby, personal communication)

Vitamin A supplementation (VAS) appears to amplify these NSE in a sex-differential manner, thus enhancing the negative effects of DTwP and the beneficial effects of live vaccines (Benn et al., 2003). VAS was associated with increased mortality for girls who

had received DTwP as their most recent vaccine (Benn et al., 2009b; Benn et al., 2009a; Benn et al., 2010). This result supports other reports that receiving DTwP after measles vaccine affects females in a negative way (Benn and Aaby, 2012). Another study in Ghana showed that girls randomised to VAS had two-and-half fold higher mortality if they received DTwP during follow-up whereas boys receiving vitamin A had lower mortality than placebo recipients (Benn et al., 2009a).

If the existence of sex-differential and non-specific effects of vaccines are proven and become accepted, then suggestions for different vaccine schedules in males and females in the future might be justified (Flanagan et al., 2011). Convincing epidemiological data from randomized trials and clarification of the mechanisms needed to convince the scientific community. Some of these studies are now emerging (Flanagan et al., 2013b).

#### **1.16 Sex differences in specific immunity to infections and vaccines**

Studies in humans and mice have demonstrated that the intensity and prevalence of infection with viruses, bacteria, and parasites are higher in males than females, which likely involves physiological as well as behavioral differences (Merkel et al., 2001; Kahlke et al., 2002). Higher innate and adaptive immune responses to pathogen challenge have been reported in adult females than their male counterparts (Klein, 2000; Klein, 2005). Cells isolated from <50 years olds and cultured with rhinovirus produced significantly higher IFN- $\gamma$  and IL-13 in women than men (Carroll et al., 2010). Production of the immunosuppressive cytokine IL-10 from adult PBMCs stimulated with TLR8 and TLR9 or viruses (influenza and Herpes-simplex-1) was significantly lower in females, alongside higher amounts of IFN- $\alpha$  after TLR7 stimulation compared to males (Torcia et al., 2012). This increased immunity in females can be beneficial for clearing infections, these immune responses can be detrimental if they become too robust or remain elevated for too long, leading to the development of immunological pathology.

There is compelling evidence that suggest that CD4+ T cell activation is different in males and females. Females lean toward a Th2 immune responses and B cell activation, while males show a more prominent Th1 CD4+ and CD8+ T cell response (Reviewed in (Pennell et al., 2012)).

A handful of studies describe male / female differences in vaccine responses. Females have more robust innate responses than males to yellow fever vaccination. Females also have higher antibody responses to the influenza vaccine, combined measles, mumps, rubella (MMR) vaccine, and hepatitis A and B vaccines (Klein et al., 2010). In a study carried out in children aged 6 months administered simultaneously with standard-titre Schwarz measles vaccine and vitamin A, females were found to be less likely to seroconvert than males (OR = 0.34) (Semba et al., 1995). These data support sex differences in response to vaccination are evident in both adults and children, although the available data remain limited.

### **1.17 Potential mechanisms for sex differences in immunity to vaccines**

Mechanistic explanations include a role for sex steroids. Thus, testosterone, oestradiol and progesterone all bind to specific receptors on immune cells including macrophages, lymphocytes and DCs (Reviewed in (Klein, 2012)). This binding activates cell signaling pathways such as NF- $\kappa$ B, cJun and interferon regulatory factor 1 (IRF-1). This leads to the production of different cytokines and chemokines (Reviewed in (Klein, 2012)). Differences in the balance of sex hormones could account for the sex differences in immune responses to vaccines in early life. Oestrogens are known to regulate Th1 and Th2 responses in females during the menstrual cycle in a bi-phasic manner. It has been reported that low-dose oestrogen during menstruation and during the luteal phase induce Th1 immune responses; on the other hand, high-doses during the follicular phase promote Th2 mediated immunity (Pernis, 2007).



There are two X chromosomes in females (XX) but one is generally silenced (inactivated) *in-vivo*; males (XY) have only one X chromosome and one Y chromosome, both being universally expressed. Since many the immune response genes are X-linked, this provides a possible explanation for sex differences in immunity to infections and vaccination (Fish, 2008). Some of the X-linked genes involved in immune responses include immune response related proteins, cytokine receptors, TLRs and transcriptional and translational effectors (Fish, 2008). These theories have never been formally studied in the context of vaccination.

### **1.18 Gene signatures after vaccination**

Systems biology is an interdisciplinary approach that systematically describes the complex interactions between all the parts in a biological system, with a view to elucidating new biological rules capable of predicting the behaviour of the biological system. Systems biology focuses on studying the structure and dynamics of the whole system (Kitano, 2002). The immune response to vaccination involves a complex network of interrelated immune pathways and yet most vaccine immunogenicity studies focus on the induction of vaccine specific antibodies or T cells (Pulendran, 2009). The immune response network theory states that the response to a vaccine is the cumulative result of genes and their interactions and is, therefore, theoretically predictable (Poland et al., 2008). The complete sequencing of the human genome along with technical advances in microfabrication has led to the development of methodologies to allow high throughput analysis of transcription profiles. Systems biology offers new and exciting potential to study the immune response to vaccination. These systems biology approaches might be used to identify the gene expression patterns or molecular signatures that are induced rapidly after vaccination. These might then be correlated with, and predict, the later development of protective immune responses (Pulendran, 2009).

Analysing the transcription profile *ex-vivo* in unstimulated whole blood or peripheral blood mononuclear cells (PBMCs) provides a 'snapshot' of the dynamics of the entire immune

system at a single point in time. If whole blood is used then genes from all cells in the blood will be represented, whereas PBMC analysis limits the information to those cell populations within the PBMC fraction; namely B cells, T cells, NK cells, monocytes, macrophages and dendritic cells (DCs). There is relatively little day-to-day background variation in global gene expression in whole blood and PBMCs from healthy individuals, although factors such as time of day, sex of the subject and age do affect the profile (Reviewed in (Flanagan et al., 2013a)). A number of studies have shown that the whole human transcriptome profile is altered in disease states such as autoimmunity, haematological malignancy, infectious diseases and solid organ transplant recipients (Chaussabel et al., 2010), however very few studies have examined the *ex-vivo* transcriptome profile to vaccination. Analysis of RNA extracted from whole blood samples collected directly into PAXgene tubes (Qiagen) has been used to study the immune response to influenza vaccine (Bucasas et al., 2011b). An alternative to the *ex-vivo* approach is to stimulate *in vitro* with vaccine antigens prior to microarray analysis (Reviewed in (Flanagan et al., 2013a)), as used to study the PBMC response to BCG vaccination (Fletcher et al., 2009). Vaccines elicit an early pro-inflammatory response mediated by innate reactivity to pattern associated molecular patterns and adjuvants contained within the vaccine. The memory T cell, B cell and humoral responses generally take several weeks to develop, the kinetics depending on the vaccine administered (Flanagan et al., 2013a). The time of sampling in these systems studies is therefore very important.

Systems biology can be used to aid the analysis of vaccine safety and immunogenicity since conventional immunological assays such as ELISPOT, antibody-ELISA, and multiplex-cytokine assays could be combined with genome-wide co-expression network analysis to provide more informative data. Only a few studies have been published using this methodology but they have shown the importance of this approach. In a recent study of global gene expression following influenza vaccination using Illumina arrays, the authors found maximal expression changes within the first 24h following vaccination,

which strongly correlated with the magnitude of antibody response (Bucasas et al., 2011a). Another group used a systems biology approach to identify molecular gene signatures that predict the magnitude of adaptive responses in humans vaccinated against yellow fever (Querec et al., 2009).

Microarray profiling of vaccinated and unvaccinated infants has enormous potential to provide answers as to how NSE / heterologous effects are mediated, and to identify biological pathways and biomarkers that might predict them. As mentioned earlier females are generally more susceptible to heterologous effects of vaccines (Flanagan et al., 2011). Females expressed far more innate immune response genes than males following yellow fever vaccination, in particular innate immune response genes (Klein et al., 2010), and a similar pattern has emerged following measles vaccination (Flanagan KL et al., unpublished data). Analysing males and females together may therefore lead to a loss of information since changes in gene expression may only become apparent when males and females are analysed separately. Thus transcriptome studies of vaccine responses should ideally be analysed separately by sex in addition to analysing all donors together.

### **1.19 Study rationale**

This study was designed as the first comprehensive randomized trial investigating the immunological mechanisms of the non-specific effects of MV and DTwP vaccination in infants. It should provide insights into why different vaccine schedules exert NSE on morbidity and mortality, dependent on sex. Our aim is to establish whether the two vaccines lead to distinct immunological footprints that could account for NSE, and whether these alter when the vaccines are combined. If the mechanisms of NSE are understood this would help guide recommendations for future vaccine combinations as well as introduction of new vaccines into the EPI. Furthermore, if proven to be true then the current EPI schedules may need to be refined.

## 1.20 Study hypotheses

In this thesis we aim to address the following hypotheses:

- Administration of MV or DTwP vaccines to 9 month old infants leads to distinct polarization of the immune profile, as determined by multiplex cytokine and flow cytometry analysis following *in vitro* stimulation with TLR agonists, and vaccine specific and non-vaccine antigens.
- MV and DTwP will elicit distinct immune response pathways as determined by whole human transcriptome microarray analysis of RNA extracted from whole blood.
- Co-administration of MV with DTwP will alter the above responses.
- Males and female infants will differ in their immunological profiles following MV, DTP and MV+DTP vaccination.
- The above methodology will identify signatures / biomarkers that could account for the increased female susceptibility to the deleterious and beneficial non-specific effects of vaccines.

## **CHAPTER 2**

### **MATERIALS AND METHODS**

## 2.1 Polychromatic flow cytometry

Polychromatic flow cytometry was performed on the 9 and 10 month blood samples from all infants.

### 2.1.1 Overnight whole blood cultures

100µl per test of heparinized whole blood was cultured overnight in a 96 well U-bottom plate with the following antigens:

- Anti-CD3 ( $\alpha$ CD3, Mouse IgG k, Clone UCHT1, 5 µg/ml; Becton-Dickinson (BD), USA) plus anti-CD28 ( $\alpha$ CD28, Mouse IgG1 k, Clone CD28.2, 5 µg/ml; eBiosciences, UK)
- Tetanus toxoid (TT, 10 µg/ml; Sanofi Pasteur)
- Purified protein derivative (PPD, 10 µg/ml; Statens Serum Institute, Denmark)
- *E. coli* K12 Lipopolysaccharide Proteins S (LPS, 1 µg/ml; InvivoGen, San Diego, California)
- Measles Haemagglutinin ("H") Protein peptide pool (15 mer overlapping peptides of > 95% purity spanning HA peptide ("H"), 1 µg/ml; Sigma-Genosys, UK). The HA peptide was used for the peptide pool, which consisted of a total of 122 peptides (Table 2.1). The lyophilized peptides were diluted in Dimethyl sulfoxide (DMSO) or RPMI 1640, Sigma-Aldrich) as necessary before pooling them. The manufacturer provided information on the weighted average, peptide hydrophobicity, and solubility. Peptides with weighted average  $\leq 0.5$  were hydrophilic and soluble; these were diluted with RPMI at 20mg/ml or 40mg/ml. Those peptides with weighted average  $\leq 1$ , were of moderate hydrophobicity and solubility (difficult to dissolve) were diluted to give a concentration of 20mg/ml or 10mg/ml. Finally, peptides with weighted average  $\geq 1$  were very hydrophobic and of very low solubility (very difficult to dissolve) were diluted DMSO to a concentration of 5mg/ml and were pooled to give a concentration of 100µg/ml. We then calculated the volume of each peptide at the above-mentioned concentrations, needed to give a final concentration of 100µg/ml for peptide pool.

- 100µl blood without antigen; medium alone was added (negative control for spontaneous cytokine production).

**Table 2.1 Amino acid sequences of 122 Measles “H” Peptides incorporated in the pool used in this study**

| Measles “H” Peptides, 15mers overlapping by 10 |                     |                     |                     |                     |
|--|---------------------|---------------------|---------------------|---------------------|
| 1 mspqrdinafykdn                               | 26 npdreydfdltwci   | 51 myrvfevgvirmpgl  | 76 lmetcfqqackgki   | 101 paevdgdvklssnlv |
| 2 drinafykdnphpkg                              | 27 ydfdrdltwcinpper | 52 evgvirmpglgapvf  | 77 cfqqackgkqalce   | 102 gdvklssnlvilpgq |
| 3 fykdnphpkgsrivi                              | 28 ltwcinnpperikldy | 53 mpglgapvfhmtny   | 78 ckgkqalcenpewa   | 103 ssnlvilpgqdlqyv |
| 4 phpkgsrivnrehl                               | 29 npperikldydqyca  | 54 gapvfhmtnyleqp   | 79 qalcenpewapikdn  | 104 ilpgqdlqyvltyd  |
| 5 srivnrehlmidrp                               | 30 ikldydqycaavae   | 55 hmtyleqpvsndls   | 80 npewapikdnripsy  | 105 dlqyvltydtsrve  |
| 6 nrehlmidrpvylla                              | 31 dqycaavaeelma    | 56 leqpvsndlsncmva  | 81 plikdnripsygvlsv | 106 latydsrvehavy   |
| 7 midrpyvlavlfvm                               | 32 dvaaelmnaalvnst  | 57 sndlsncmvalgelk  | 82 ripsygvlsvdlsit  | 107 tsrvehavyvyysp  |
| 8 yvlavlfvmflsli                               | 33 elmnaalvnstletr  | 58 ncmvalgelklaalc  | 83 gvlsvdlsitvelki  | 108 ehavyvyyspsrfs  |
| 9 vlfvmflsliagil                               | 34 lvnstletrtnqf    | 59 lgelklaalchreds  | 84 dlsitvelkikiasg  | 109 yyspsrfsyfyf    |
| 10 flsliagilagirl                              | 35 llettrtnqflavsk  | 60 laalchredsitipy  | 85 velkikiasgfgpli  | 110 srfs yfypfrlpik |
| 11 gliaagirlhraai                              | 36 tttnqflavskgncsg | 61 hredsitiipyqsgsk | 86 kiasgfgplithgsg  | 111 yfypfrlpikgvpie |
| 12 agirlhraaiytaei                             | 37 lavskgncsgpttir  | 62 itipyqsgskgvsfq  | 87 fgplithgsgmdlyk  | 112 rlpikgvpieqvec  |
| 13 hraaiytaeihksls                             | 38 gncsgpttirgqfsn  | 63 qsgskgvsfqlvklg  | 88 thgsgmdlyksnhnn  | 113 gvpieqvecftwdq  |
| 14 ytaeihkslstndlv                             | 39 pttirgqfsnmsls   | 64 gvsvqlvklgwwksp  | 89 mdlyksnhnnvywti  | 114 lqvecftwdqklwcr |
| 15 hkslstndlvtnsie                             | 40 gqfsnmslsldlyl   | 65 lvklgwwksptdmqs  | 90 snhnnvywtippmk   | 115 ftwdqklwcrhcvl  |
| 16 tndlvtnsiehqvd                              | 41 mslsldlylgrgyn   | 66 vwkspdmqswvtls   | 91 vywtippmk nlaig  | 116 klwcrhcvlades   |
| 17 tnsiehqvkdvltp                              | 42 ldlylgrgynvssiv  | 67 tdmqswvtlsddpv   | 92 ppmknlalgvintl   | 117 hfcvldesegghit  |
| 18 hqvkdvltpfkiiig                             | 43 grgynvssivtmtsq  | 68 wvtlsddpvidrly   | 93 nlalgvintlewi    | 118 adsesgghithsgmv |
| 19 vltplfkiiigdevgl                            | 44 vssivtmtsqgmygg  | 69 tddpvidrlysshr   | 94 vintlewi         | 119 gghithsgmvmgvs  |
| 20 fkiigdevglrtpr                              | 45 tmtsqgmyggtylve  | 70 idrlysshrvgiad   | 95 ewiprkvspylfnv   | 120 hsgmvmgvsctvtr  |
| 21 devglrtprftdlv                              | 46 gmyggtylvekpnl   | 71 lsshrvgiadnqakw  | 96 kvspylfnvpik     | 121 gmvsctvtrdgt    |
| 22 rtprftdlvkfisd                              | 47 tylvekpnlsskrse  | 72 gvianqakwvaptt   | 97 ylnvpik          | 122 ctvtrdgt        |
| 23 ftldvkfisdikifl                             | 48 kpnlsksrslsqsl   | 73 nqakwvapttrddk   | 98 pikeagedchaptly  |                     |
| 24 kfisdikiflnpdre                             | 49 skrselsqslmyrvf  | 74 avpttrddklmet    | 99 gedchaptlypaevd  |                     |
| 25 kikflnpdreydfdr                             | 50 lsqslmyrvfevgvi  | 75 rtdklmetcfqqa    | 100 aptlypaevdgvkl  |                     |

Plates were sealed and incubated for 2 hours at 37°C, 5% CO<sub>2</sub>. Brefeldin A (Sigma-Aldrich, UK) was then added at a final concentration of 10µg/ml to all stimulated wells and further incubated for 16 hours. An unstimulated well was used to control for background cytokine production (negative control). For each experiment, unstimulated control cells were used to set up compensations (50µl for single stains and 100µl for unstained) (Table 2.2).

**2.1.2 Intracellular cytokine staining procedure**

**2.1.2.1 Conjugated antibodies used**

All antibodies were titrated in preliminary assays to determine the volume required for optimal staining as indicated in Tables 2.2 and 2.3.

**Table 2.2 Flow cytometry antibodies used for compensation**

| Marker | Clone                      | Source        | Fluorochrome | Vol/test (µl) |
|--------|----------------------------|---------------|--------------|---------------|
| CD3    | Mouse IgG2a k, Clone HIT3a | BD Pharmingen | FITC         | 2             |
| CD3    | Mouse IgG2a k, Clone HIT3a | BD Pharmingen | PE           | 2             |
| CD3    | Mouse IgG1 k, Clone SK7    | BD Pharmingen | PerCP        | 2             |
| CD3    | Mouse IgG1 k, Clone UCHT1  | e-Biosciences | PE-Cy7       | 1             |
| CD3    | Mouse IgG1 k, Clone UCHT1  | BD Pharmingen | Pacific Blue | 1             |
| CD3    | Mouse IgG2a k, Clone HIT3a | BD Pharmingen | APC          | 5             |
| CD3    | Mouse IgG1 k, Clone SK7    | BD Pharmingen | APC-Cy7      | 2             |

CD - cluster of differentiation  
FITC - fluorescein isothiocyanate,  
PE - phycoerythrin  
PerCP - peridinin chlorophyll protein  
APC - allophycocyanin  
IgG - immunoglobulin  
Vol/test (µl) - denote antibody volumes used per test

**Table 2.3 Flow cytometry antibodies used for surface staining and ICS**

| Marker | Clone                           | Source                 | Fluorochrome | Vol/test (µl) |
|--------|---------------------------------|------------------------|--------------|---------------|
| IL-13  | Mouse IgG1, clone PVM13-1       | e-Biosciences          | FITC         | 0.5           |
| IL-2   | Rat anti-human, clone MQ1-17H12 | Beckton Dickinson (BD) | PE           | 2             |
| CD8    | Mouse IgG1, clone SK1           | Beckton Dickinson (BD) | PerCP        | 5             |
| CD56   | Mouse IgG1 k, clone B159        | Beckton Dickinson (BD) | PE-Cy7       | 2             |
| IL-10  | Rat IgG1 k, clone JES3-9D7      | e-Biosciences          | Pacific Blue | 2             |
| IFN-γ  | Mouse IgG2b, clone 25723.11     | Beckton Dickinson (BD) | APC          | 1             |
| CD4    | Mouse IgG1 k, clone RPA-T4      | Beckton Dickinson (BD) | APC-Cy7      | 1             |

IL - interleukin  
CD - cluster of differentiation  
FITC - fluorescein isothiocyanate,  
PE - phycoerythrin  
PerCP - peridinin chlorophyll protein  
APC - allophycocyanin  
IgG - immunoglobulin  
Vol/test (µl) - denote antibody volumes used per test

**2.1.2.2 Surface staining of cells**

After 16 hours of culture, cells were lysed with 200µl RBC lysing buffer (Fluorescence Activated Cell Sorter (FACS) lysing buffer reconstituted to 10x original volume with distilled water, BD Biosciences) for 10 min at room temperature (RT). The plate was then



centrifuged at 2,000rpm for 10 mins, washed in FACS buffer (0.5% Bovine Serum Albumin, 0.1% Ethylenediamine tetra-acetic acid (EDTA) 0.1% sodium azide (NaN<sub>3</sub>) in phosphate buffered saline (BSA) for 5 mins before being stained with surface antibodies.

Cells were incubated with 30µl of surface antibody cocktail (CD4, CD8, CD56) (Table 2.3) for 30 minutes at 4°C in the dark (plate was covered with foil). The plate was then spun at 2,000 rpm for 5 mins. The supernatants were flicked off, and the plate gently vortexed. The cells were resuspended and washed with 200µl FACS buffer. Compensations were done electronically for every experiment in order to minimize spectral overlap for each photomultiplier on the CyanADP® Flow cytometer (Beckman-Coulter, USA).

#### **2.1.2.3 Staining for intracellular cytokines**

FACS lysing buffer contains fixative, so following this step, cells were permeabilised by adding 200µl of Cytofix/Cytoperm solution (BD, USA) and incubating for 20 mins at 4°C. The plate was then centrifuged at 2,000 rpm for 5 mins and supernatants discarded. 10µl of ICS cocktail containing IL-13, IL-2, IL-10 and IFN-γ was added to each well (see Table 2.3 for amounts used per antibody) and incubated for 30 mins at 4°C. After the incubation time, 200µl of Perm/Wash (BD) was added and the plates centrifuged at 2,000 rpm for 5 mins. Supernatants were discarded and the wash repeated a second time. Supernatants were again discarded and 150µl FACS fix (1% Paraformaldehyde (PFA)) was added to each well. Cells were transferred to 5ml round bottom tubes (BD Falcon, Cedex, France) before acquiring on the flow cytometer.

#### **2.1.3 Cell Acquisition**

Cells were acquired on a CyAnADP flow cytometer (Beckman-Coulter, USA) (CyAn). Following compensations with each fluorochrome, we collected a minimum of 100,000 lymphocyte events for each sample after gating on FSC/SSC.

#### **2.1.4 Data Analysis by Flowjo**

Data were analyzed using Flowjo software ([www.flowjo.com](http://www.flowjo.com)). The gating strategy is discussed in detail in chapter 7.

### **2.2 Bio-plex analysis for multiple soluble cytokines**

#### **2.2.1 Antigen stimulation**

These cultures were set up exactly the same as in the overnight cultures for ICS detailed above except that Brefeldin A was not added (because it blocks cytokine secretion from the Golgi apparatus). After overnight culture, plates were centrifuged at 2,000 rpm for 5 mins and 50µl supernatant collected from each well into pre-labelled tubes. These were stored at -20°C for later analysis.

#### **2.2.2 Cytokine multiplex assay procedure**

##### **2.2.2.1 Samples, standards and controls**

The Bio-Plex 200 Suspension Array system (Bio-Rad Laboratories, France) was used for analysis of cytokines in culture supernatants. Supernatants that were collected from the overnight cultures were thawed and centrifuged at 1,200 rpm for 2 minutes to pellet out any debris so as to avoid blockage when acquiring samples.

25µl of culture supernatants were plated out in a 96 well plate in preparation for the assay. All samples were diluted 1:2 with media containing 10% serum before running the assay in order to detect the secreted cytokines within the range of the standard curves.

We initially ran a series of Group 1 (MV) and Group 2 (MV+DTwP) 10 month samples using a 27-plex cytokine kit in order to identify the key cytokines / chemokines that were altered in the combined vaccine group (Group2) compared to the MV group (Group 1), and those that differed between males and females. The 27-plex kit consisted of pre-mixed beads specific for: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, Eotaxin, FGF basic, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1a, MIP-

1b, PDGFbb, RANTES, TNF- $\alpha$  and VEGF. The 10 cytokines/chemokines that were predominantly different between vaccine groups were selected and used to analyse the remainder of the 9 and 10 month culture supernatants. The 10 cytokines selected were: IL-4, IL-1 $\beta$ , IFN- $\gamma$ , IL-10, IL-12(p70), Eotaxin, GM-CSF, PDGFbb, TNF- $\alpha$  and VEGF; incorporating a combination of Th1, Th2 and innate cytokines (Table 2.4) below.

**Table 2.4 10-Plex cytokines analysed, functions and low-high standard value across all plates**

| Analyte                                    | Abbreviation  | Function   | Low std (pg/ml) | High std (pg/ml) |
|--|---------------|--|-----------------|------------------|
| Interleukin-4                              | IL-4          | Th2 cell-mediated immunity, IgE class switching in B cells, Inhibits differentiation of Th1 cells                          | 0.125           | 32768            |
| Interleukin-1 $\beta$                      | IL-1 $\beta$  | Initiation of acute-phase responses  | 2               | 32768            |
| Interleukin-10                             | IL-10         | Auto-regulator of Th1 cell activation, anti-inflammatory, Th2 cytokine, can inhibit Th1 responses but not just that.       | 2               | 32668            |
| Interleukin-12(p70)                        | IL-12(p70)    | Regulation of inflammatory response, Mediates T cell-dependent immunity  | 1               | 49152            |
| Eotaxin                                    | Eotaxin       | Development of allergic responses  | 0.1875          | 32768            |
| Granulocyte, macrophage stimulating factor | GM-CSF        | Promotes proliferation, activation, differentiation of macrophages, granulocytes, neutrophils, eosinophils, and monocytes. | 0.125           | 32768            |
| Interferon-gamma                           | IFN- $\gamma$ | Immunomodulatory effects, inhibits Th2 lymphocyte proliferation, anti-viral and anti-parasitic agent                       | 1               | 32768            |
| Platelet-derived growth factor-bb          | PDGF-bb       | Promotes lymphangiogenesis (Cao R et al. 2004)   | 0.75            | 32768            |
| Tumour necrosis factor- $\alpha$           | TNF- $\alpha$ | Cellular proliferation/differentiation, Th1 and innate cytokine tumorigenesis, apoptotic or necrotic cell death            | 0.5             | 65536            |
| Vascular endothelial growth factor         | VEGF          | Vascularization, promotes cell survival  | 0.5             | 49152            |
| Std - Standard                             |               |  |                 |                  |

The lyophilized standard was reconstituted in 500 $\mu$ l RPMI medium containing 10% bovine serum albumin (BSA) (VWR, UK), vortexed and incubated on ice for 30 minutes. The manufacturer's instructions were followed in making 1:4 serial dilutions of the standard using the RPMI medium and thus creating 8 standards for the low Photomultiplier Tube (PMT) settling, allowing for a broad range standard curve. Eight 1.5ml eppendorf tubes were labelled (S1 - S8); 72 $\mu$ l of standard diluent was added to S1 tube and 150 $\mu$ l to the rest of the tubes (S2 - S8). 128 $\mu$ l of the reconstituted standard was added to the S1 tube. The tube was vortexed and 50 $\mu$ l from this S1 tube was added to the next tube (S2). 50 $\mu$ l from this S2 tube was added to the S3 tube and so on making serial dilutions of the standard. The pipette tip was discarded after each transfer. The standard ranges differed for each analyte and are listed in Table 2.3.

The reagent and wash buffer solutions in the 96 well plate were removed during all steps of the assay by vacuum filtration using the Aurum vacuum manifold (BioRad, Belgium) with 1-2 Hg pressure. A lint free paper towel was used to blot excess fluid from the bottom of the plate after each vacuum step.

#### **2.2.2.2 Assay protocol**

The filter plate provided with the kit was first pre-wet using 100µl of assay buffer (Bio-Rad Laboratories, France). The assay buffer was removed by vacuum filtration and the plate was washed twice with 100µl wash buffer.

The conjugated microsphere beads were then prepared: pre-mixed beads were vortexed vigorously for 20-30 seconds to dislodge bead aggregates before diluting with assay buffer (according to manufacturer's instructions) and 50µl was added to each well of the assay plate. 50µl samples (single wells), 50µl standards (induplicate) and 50µl RPMI (Blank, added to two wells to measure background production of cytokine) were added to the appropriate wells. The plate was sealed with sealing tape, covered in foil and incubated by shaking at 1,100 rpm for 30 seconds followed by 300 rpm for 30 minutes.

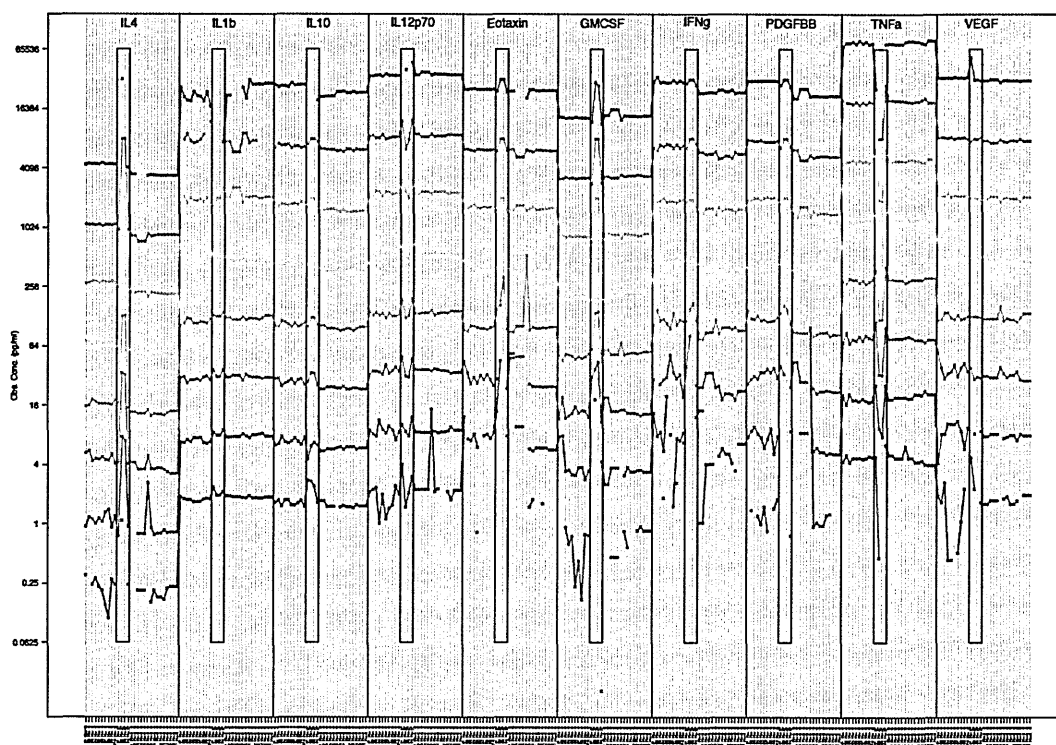
The biotinylated detection antibody was diluted according to the manufacturer's instructions during this incubation time and wrapped in foil. At the end of the incubation, the plate was washed three times with 200µl per well of wash buffer with the vacuum manifold. Following blotting, the detection antibody was vortexed gently and 25µl added to each well. The plate was sealed with sealing tape, covered in foil and incubated for 30 mins. The streptavidin-PE reporter antibody was diluted 1:10, 25µl added to each well of the assay plate (following washing) and incubated (shaking) for 10 minutes. The plate was given a final three washes with wash buffer and each well resuspended in 125µl of assay buffer. The plate was then shaken for 30 secs at 1,100 rpm before reading it on the Bio-

Plex 200 Suspension Array system (Bio-Rad) with Bio Plex Manager software version 4.1.1 (Bio-Rad Laboratories, Hercules, CA).

### **2.2.3 Calibration and acquisition**

The Bio-Plex reader was calibrated before every run using CAL1 and CAL2 calibration kits (Bio-Rad, UK) with low RP1 target values (used for broad range standard curves).

Data acquisition was set for 50 beads per region and the bead map at 100 regions. The sample volume was set to 50 $\mu$ l and the DD gate value was set at 5,000 (low) and 25,000 (high). Following data acquisition, normalization of the data was performed including removing standard curve outliers with a co-efficient of variation (%CV) greater than 10% and observed/expected ratio  $\times 100$  (obs/exp\*100) outside the range of 100  $\pm$  20. The MFI max (FL-Bkgd) of the individual standard curves was defined as the FI-Bkgd just before the non-linear part of the curved is reached. The checked/corrected bioplex data was exported as an excel file. All results had background wells subtracted automatically. Standard concentrations were fairly similar across all plates (Figure 2.1).



**Figure 2.1 Per-plate standard curve concentrations.** Standard curves for all cytokines for all plates with the observed concentrations in pg/ml indicated on the Y-axis and all plates on the X-axis. Each colour represents one of the 8 standard dilutions/concentrations (ranging from 0.25 pg/ml to 4096 pg/ml). For each cytokine, the observed concentration for each of the 29 experiment plates is plotted. The light-grey vertical rectangles highlight data coming from the 27-Plex plates. Graphs prepared by Thorsten Foster at the Department of Pathway Medicine, University of Edinburgh.

#### 2.2.4 Data analysis

Antigen-stimulated wells had the medium control background value subtracted. Results that were out of range were corrected (>OOR or <OOR). Samples >OOR were corrected as two times the highest value read for that particular cytokine and samples <OOR were corrected as half the lowest value for that particular cytokine before statistical analysis.

### 2.3 Diphtheria, tetanus and pertussis antibody measurements using a multiplex immunoassay (MIA)

*These assays were kindly performed by our collaborators based at the National Institute of Public Health and the Environment (RIVM), Netherlands. This technology has now been successfully transferred to MRC, The Gambia as a result of this collaboration.*

Samples assayed were 50µl aliquots of plasma from 9 month (pre-vaccination) and 10 month (1-month post-vaccination) old donors. Multiplex technology can be used for the detection and quantification of multiple vaccine antibodies in relevant samples (Pickering et al., 2002a; Pickering et al., 2002b; Lal et al., 2004; Lal et al., 2005; Prince et al., 2006; Pickering et al., 2007). It allows a higher sample throughput using a small sample volume without losing the advantages of the conventional ELISA assay. The multiplex microsphere based fluorescent immunoassay quantifies total IgG serum antibodies directed against the target antigens: pertussis toxin (Ptx), diphtheria toxoid (Dtx) and tetanus toxoid (Ttx). Dtx, Ttx and Ptx standards and controls were prepared in-house (reviewed in (van Gageldonk et al., 2008)). When diluted human serum is added to these beads, antibodies specific for Ptx, Dtx and Ttx bind to the antigen proteins. The bound antibodies were detected using a goat anti-human conjugate labeled with R-phycoerythrin. The beads are individually measured using a Bio-Plex 200 system.

### **2.3.1 Conjugation of Ptx, Dtx and Ttx to carboxylated microspheres**

The two solutions that make up the bead activation buffer were prepared separately and were combined just before use. This bead activation buffer contains 1 x phosphate buffered saline (0.01M; pH 7.2), 2.5 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), and 2.5 mg/ml N-hydroxy-sulphosuccinimide (Sulpho-NHS). The microsphere stock solutions were vortexed vigorously for 30 mins on a minishaker. 1ml of the homogeneous carboxylated microsphere solution ( $12.5 \times 10^6$  microspheres) was added to a 1.5ml reaction vial (Starlab, Ireland) and centrifuged for 4 mins at full speed in the minispin centrifuge (13,400 rpm). The supernatant was discarded and the beads resuspended in 1ml of freshly prepared bead activation buffer and vortexed vigorously to mix. The tube was then wrapped in aluminium foil and incubated for 20 mins at room temperature (RT) under constant rotation on a rotator. The microspheres were then washed by centrifuging at 13,400 rpm for 4 mins. The supernatant was carefully removed because the microspheres easily come loose. The microspheres were resuspended in 1ml of buffer (1xPBS) and vortexed briefly. This wash step was repeated 2 more times.

While performing these washing steps, the antigen solutions for the conjugation step were prepared according to the bead conjugation ratio schedule given in Table 2.5 below.

**Table 2.5 Bead conjugation ratios used in the multiplex MIA assay**

| <b>Bead conjugation ratios:</b>  |
|--|
| Ptx (60): 10 µg/12.5*10 <sup>6</sup> microspheres<br>16 µl 0.806 µg/µl PEG004 batch + 628 µl 1xPBS: 500 µl for re-suspension of the beads  |
| Dtx (45): 50 µg/12.5*10 <sup>6</sup> microspheres (before conjugation purified diphtheria toxin (1 mg/vial = 1 mg/ml when reconstituted in 1 ml sterile water) is dialyzed 2x against 2 ltr 1xPBS).<br>50 µl 1 µg/µl Dtx batch (1 mg/ml, D05604) + 450 µl 1xPBS: 500 µl for re-suspension of the beads |
| Ttx (28): 50 µg/12.5*10 <sup>6</sup> microspheres (for conjugation purified tetanus toxin (25 µg/vial = 0.1 mg/ml when reconstituted in 250 µl sterile water) is used).<br>50 µg freeze-dried Ttx (Sigma Aldrich T3194) + 500 µl sterile water: 500 µl for re-suspension of the beads                  |

Ptx = pertussis toxin, Dtx = diphtheria toxoid and Ttx = tetanus toxoid

The protein used for coupling must be free of sodium azide, BSA, glycine, Tris or amine-containing additives and must be suspended in PBS, pH 7.4. The supernatant was then aspirated until all the remaining fluids were removed and the microspheres were resuspended in the 500µl antigen (bead conjugation ratio according to schedule above). The tube was vortexed and wrapped in foil and incubated at RT for 2 hrs under constant rotation. After 2 hrs of incubation, the microspheres were washed by centrifugation as described above, the washing step was repeated two more times. The microspheres were centrifuged at 13,400 rpm for 4 mins and the supernatant aspirated. The microspheres were then resuspended in 1ml bead storage buffer (1xPBS, 0.05%(w/v) azide, 1.0%(w/v) BSA) and vortexed at high speed for 30 secs and sonicated for 30 secs.

10µl of the homogenous aliquoted bead suspensions were diluted 1:20 in storage buffer. 10µl of the diluted microsphere solution was added to a colony counting chamber (glasstic slides Hycor) and the number of microspheres counted in 15 grids in triplex. The count was recorded, as it was required for the preparation of the bead solution in the multiplex



immunoassay. The microspheres were then stored in the dark at 4°C until required for the immunoassay. These are stable for up to 12 months.

### **2.3.2 Determination of the DTP MIA values**

Those values that were out of range, were corrected (>OOR or <OOR). Samples >OOR were deleted and samples <OOR or with MFI's below the lowest concentration of the standard curves were corrected to the established lower level of quantification (LLOQ) for statistical analysis. LLOQ = 1 EU/ml for all Ptx (established with Pertussis standard mBP: 6 steps 4-fold dilutions (1/200 to 1/204,800); LLOQ = 0.001 IU/ml for DTx (established with DT standard Bui: 8 steps of 4-fold dilutions (1/50 to 1/819,200)). For each analyte, median fluorescent intensity (MFI) was converted to EU/ml or IU/ml by interpolation from a 5-parameter logistic standard curve (log-log) for every bead region/standard.

Results marked outside the linear part (>MFI max) of the standard curve (1/200 dilution) were accepted when the %CV <20, otherwise these results were discarded and the final result is based on the 1/4,000 result only.

### **2.4 Measles HAI antibody assay**

*This assay was performed in The Gambia with the help of Jainaba Njie-Jobe.*

Plasma samples stored at -20°C were thawed and 40µl of each sample was aliquoted into labelled 1.5ml eppendorf tubes and decompemented by incubating in a water bath at 56°C for 30 mins. A 5% solution of *Cercopithecus* rbc's, diluted in HAI buffer, was prepared and 100µl aliquoted into 0.5ml eppendorf tubes and spun at 6,500 rpm in a microcentrifuge for 1-2 mins. The supernatant was discarded and the red cell pellet was retained. 35µl of the decompemented samples were then added to the pellet in the eppendorf tubes and mixed gently using a pipette before overnight incubation at 4°C. The following morning, the tubes were spun at 6,500 rpm for 2 mins to pellet the erythrocytes. 25µl of HAI buffer was then added to all wells of the microtitre plate using a multichannel pipette. Plasma (25µl) from the eppendorf tubes containing the pelleted erythrocytes was carefully added to the

appropriate well of the microtitre plate. A multichannel pipette was then used to make a 2-fold serial dilution by taking 25µl from the wells in the first column and adding it to the wells in the second column. The well contents were mixed thoroughly after each transfer and the remaining 25µl after the last transfer was discarded. 25µl of the measles antigen (diluted 1:600 in HAI buffer) was then added to all wells except the negative control wells and the sides of the plate gently tapped to ensure maximum contact. The plate was then incubated at 37°C for 3 hours. 25µl of a 0.5% solution of *Cercopithecus* rbc's (diluted in HAI buffer) was then added to all wells and the sides tapped gently to mix. The plates were left at room temperature overnight to be read the following morning. Controls were added to the assay and were treated the same way as the samples. For positive control wells, 25µl HAI buffer plus 25µl of measles antigen plus 25µl of 0.5% *Cercopithecus* rbc's was used. For the negative control wells, we added 50µl of HAI buffer plus 25µl of 0.5% *Cercopithecus* rbc's. Measles antigen was not added to the negative control wells. In-house standards were also included in this assay. The standard was serum decompemented and absorbed against *Cercopithecus* rbc's. It had been calibrated against the second international standard antisera and found to contain 500mIU/ml of measles HAI antibody. 25µl of the standard was diluted 1:4 in HAI buffer and added to the first column of the 3 standard control wells and treated the same as the samples.

#### **2.4.1 HAI plate reading**

The plates were read the next day by placing over a light box by two independent observers. The titre was given as the dilution at which no agglutination occurred, i.e there was complete neutralization of the agglutinin. The minimum detection level of the assay is 31.2 mIU. Results were expressed as log<sub>2</sub> units. A protective level is defined as 125mIU antibody or a log<sub>2</sub> titre ≥ 3 (Samb et al., 1995). Measles HAI antibody titres have been found to correlate with neutralizing antibody titres as described (Samb et al., 1995).

## **2.5 Whole-human genome microarray analysis**

*This part of the project was carried out in collaboration with the Division of Pathway Medicine, University of Edinburgh. Much of the work in Edinburgh was carried out by my collaborators although I had the opportunity to visit the unit and learn the assay and software used for analysis of the microarray data but i did not carry out the work.*

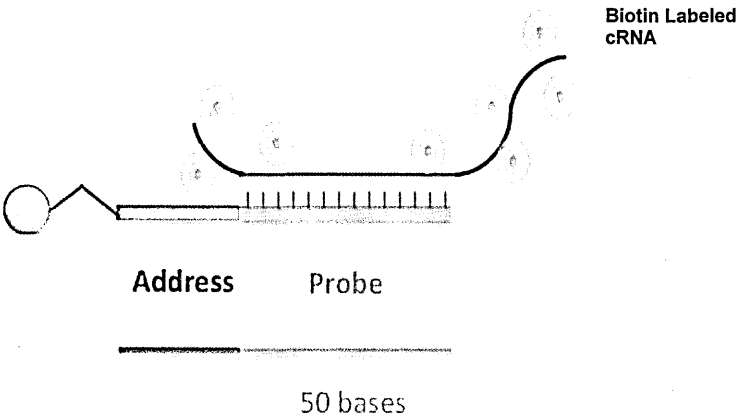
### **2.5.1 Samples for RNA extraction**

500µl whole blood collected directly into Paxgene™ tubes (Qiagen, Crawley, UK) and frozen at -70°C were thawed at room temperature for 12 hours before RNA extraction. RNA extraction was carried out using PAXgene™ blood RNA extraction kits (Qiagen) according to the manufacturer's instructions. Briefly, cells were pelleted by centrifugation at 8,050 rpm for 10mins, and pellets washed and resuspended in lysis buffer, incubated with proteinase K and binding buffer and then transferred to the shredder spin column. The flow through was mixed with ethanol and loaded on to PAXgene RNA spin columns. After a series of washing steps the DNA was degraded using DNase and the remaining RNA eluted with elution buffer and stored at -70°C. For RNA quantification a 1µl sample was loaded on to The Thermo Scientific NanoDrop™ 1000 Spectrophotometer, and the RNA concentration calculated and contamination assessed using the 260/280 absorption ratio. These steps were carried out in The Gambia and the RNA was then shipped to DPM in Edinburgh on dry ice. The RNA was subjected to a second quality analysis on arrival in Edinburgh using the Agilent Bioanalyser system (Agilent). Quality control using the Agilent Bioanalyser 2100 and Nanodrop ND1000 was used to determine RNA samples of sufficient quality and quantity, and 360 samples were selected for subsequent microarray analysis. A gel matrix was prepared according to Agilent Technologies manufacturer's handbook by centrifugation of 30µl of gel and 2µl of dye mix. Nine microliters of gel was loaded onto a Caliper®, LabChip® under pressure to fill the chip capillaries, 1µl of sample was then loaded into each well and 1µl of known marker loaded to the ladder well. The Agilent 2100 Bioanalyzer was used to process the chips and record the RNA concentration. The RNA

integrity number (RIN) was used to evaluate RNA degradation according to the 18s/28s ratio, with a target RIN of >7.

### 2.5.2 Whole Human Genome Microarray Analysis

100ng total RNA was converted to double-stranded cDNA, followed by an amplification step (*in vitro* transcription) to generate labelled cRNA, using the Ambion Illumina TotalPrep-96 RNA Amplification Kit. This produces an amplified pool of biotin-labelled cRNA corresponding to the polyadenylated (mRNA) fraction. The cRNA was quantified using OD (nanodrop). The cRNA was normalised and hybridised onto the Illumina HT12 arrays for 14-20 hours at 58°C (Figure 2.2). The unhybridized and non-specifically hybridized cRNA was washed away. The arrays were stained with Cy3-Streptavidin to bind to the analytical probes that have been hybridized to the array. This allows for differential detection of signals when the arrays are scanned. The Illumina IScan scanner is a two-colour laser (532 nm/658 nm) fluorescent scanner with a 0.53 µm spatial resolution capable of exciting the fluorophores generated during the staining step of the protocol. Light emissions from these fluors were then recorded in high-resolution images of the Array sections. The intensities of the images were extracted using Genome Studio (2010.3) Gene Expression Module (1.8.0) software.



**Figure 2.2** Hybridization of probe to a labeled nucleic acid derived from total RNA. Conversion of total RNA to double-stranded cDNA followed by amplification to produce several copies of the transcript creating a pool of biotin-labelled cRNA that correspond to the polyadenylated (mRNA) fraction.

### **2.5.3 Microarray data processing and statistical analysis**

Primary data were obtained from Tepnel/Gen-Probe (Experiment TPS01421). Arrays were all quality control tested before proceeding with the next step. The arrays used in the experiment were Human HT-12 V3 (Illumina), each comprising 47,293 features. A total of 360 arrays were QC analyzed using the array Quality Metrics package in Bioconductor (Kauffmann et al., 2009). The samples used consisted of 202 gender-matched donors with pre- and post-vaccination RNA samples available. Arrays were scored (outliers identified) on the basis of 2 metrics, namely maplot, boxplot. Raw data were transformed using a variance stabilizing transformation (VST) method prior to normalization across all arrays using the robust spline normalization (RSN) method. Expression measures (summarized intensities) are in log base 2. As gender information for each sample was provided, a "gender check" was performed, using Y-chromosome specific loci to identify male samples.

Twenty-four comparisons, manually chosen to explore the data, were undertaken using linear modeling using linear models for Microarray Data (Limma), which is an R package for statistical computing. The first step in fitting a model is to form an appropriate design matrix for the RNA sources. This requires one or two matrices to be formed; one is called the design matrix that identifies the RNA sources and the other is the contrast matrix that identifies the comparisons to be made between the RNA samples. The design matrix specifies the model. Subsequently, empirical Bayesian analysis was applied (including vertical (within a given comparison) p-value adjustment for multiple testing, which controls for false discovery rate). Multiple samples from the same individual were taken into account when modeling the data. The resulting gene lists were fully annotated and sorted in order of decreasing significance.

### **2.5.4 Functional analysis of significant genes**

Both unpaired and paired analyses were carried out since not all 9 month samples had a corresponding 10 month sample and vice versa. An overview of the underlying biological changes occurring within each paired and unpaired comparison was obtained by functional

enrichment analysis. This was performed using 2 software tools, namely KEGG pathway ([www.genome.jp/kegg/](http://www.genome.jp/kegg/)) membership and Gene Ontology (GO) terms([www.geneontology.org](http://www.geneontology.org)). Although there is always a degree of overlap between functional annotations from different sources, each set has information/applications not available in the others, and thus it is generally beneficial to consider more than one. The level of statistical significance for functional analysis was chosen to be the level at which 1% of the array features were, on average, significant. For the current dataset, raw  $p < 0.01$  was appropriate for the 18 comparisons performed.

For KEGG analysis significant genes (raw  $p < 0.01$ ) from each comparison were analyzed for enrichment of KEGG pathway membership using a hypergeometric test. Enrichment ( $p < 0.05$ ) was assessed for up-regulated and down-regulated genes separately.

The GO project uses three ontologies for the description of genes and their associated biological processes, cellular components and molecular functions independent of the species. This ensures uniformity of queries across databases. Significant genes (raw  $p < 0.01$ ) from each comparison were analyzed for enrichment of GO terms across all three GO ontologies using a hypergeometric test. Enrichment ( $p < 0.001$ ) was assessed for up-regulated and down-regulated genes separately.

### **2.5.5 Network analysis of the dataset**

A data matrix of normalized, anti-logged, intensity values for probesets on all arrays was generated. The Pearson correlation between every object (a single probeset's values across all arrays) and every other object in that matrix was determined, effectively calculating the similarity in the profile of expression of every gene on the array across the samples analyzed, to every other gene on the array. All relationships above a certain threshold were stored.

The stored annotated data was used as an input file for the generating networks using Biolayout software (available at [www.biolayout.org](http://www.biolayout.org)). Networks were laid out for all

relationships exhibiting a Pearson correlation greater than 0.75. Markov clustering (MCL) of nodes was undertaken (expansion value 1.7), and functional analysis of each cluster was performed using both public and proprietary expert knowledge.

Expression networks were constructed employing the Ingenuity Pathway Analysis software (Ingenuity Systems, ([www.ingenuity.com](http://www.ingenuity.com))). Illumina Probe IDs were imported into the Ingenuity software and mapped to the Gene Symbol from Ingenuity database. Genes that had adjusted p-value, 0.05 and associated with a canonical pathway in Ingenuity's Knowledge Base were used for pathway analysis.

## **2.6 Statistical analysis of cellular assays and antibody data**

### **2.6.1 Basic analysis of cellular and antibody data**

Most of the cellular and antibody data generated in this study were non-normally distributed and thus non-parametric tests were used throughout using Prism statistical software. Comparisons between males and females, and responses between vaccine groups were generally performed using the Mann-Whitney U-test at 5% significance level and differences across all groups were analysed using the Kruskal-Wallis test with Dunn's multiple comparison testing. The Benjamini-Hochberg False Discovery Rate was used to correct for the many comparisons made (henceforth referred to as FDR) (Benjamini and Hochberg, 2005). The FDR accounts for multiple adjustments when more than 1 parameter is being measured in an assay. It controls the false discovery rate, that is, the expected proportion of false positives among the variables for which there is a significant difference. This means that the proportion of true null hypotheses is lower than a specified threshold. For our 10 cytokines, P-values  $<0.035$  were considered significant to account for FDR.

### **2.6.2 GLS regression model**

Development of a model for the soluble cytokine data was done under the guidance of David Jeffries and Lindsey Kendall at the Statistics department, MRC Gambia unit.

Other statistical tools for the Bioplex cytokine data analysis employed a GLS regression model using STATA statistical software, STATA 12.1, Copyright 1985-2011 StataCorp LP, Statacorp USA, (<http://www.stata.com>). Since our data were non-normally distributed, they were first ranked to make them fairly normal. Interactions between bleed and group by sex, as well as sex and bleed by group, for all conditions were performed. In order to do this, we first had to write a model that fit the data and described it well.

We first set STATA to handle the data by defining the entity (donor) in the dataset, using the command `xtset donor`. We built the model by using commands that would test for interactions between parameters (bleed, group, sex) for all the conditions in the data at each time point. The model was written as; `xtreg r10 i.bleed##i.group i.bleed##i.sex2 i.sex2##i.group if condition2=2`, for condition 2 in this example testing males versus females for each bleed group comparison. The model was used for each condition and all our analysis was derived from it. We compared cytokine levels of the three vaccine groups at 9 months and at 10 months. We also assessed whether being male or female (effect of sex) affected cytokine levels in a group. To study sex differences, we directly compared males and females within each group at 9 months and at 10 months.

I was not able to do a pre to post comparison for this chapter because it is very difficult and cumbersome to interpret a three way comparison, so we did a two way comparison for each time point. A pre to post comparison has been done for all the other chapters in the thesis. The `lincom` command (linear combination) was used to calculate linear combinations of the parameters of our linear model. This `lincom` command was used after fitting the model to run all our analysis to obtain the P-values, confidence intervals, standard errors and coefficient of variation.



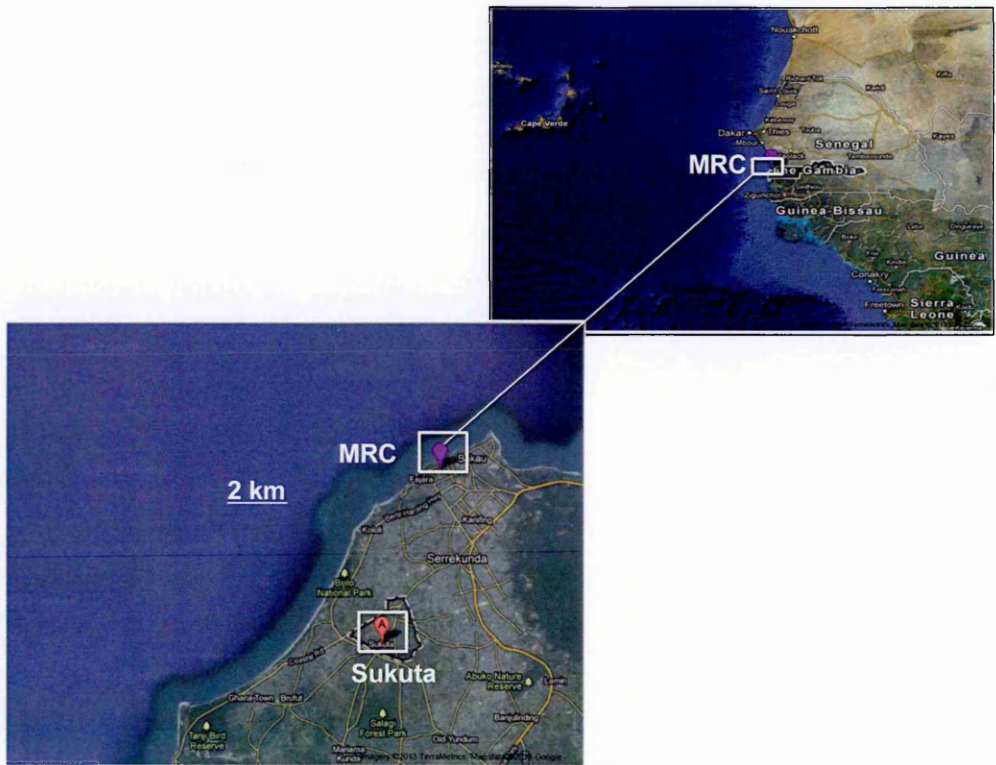
**CHAPTER 3**

**STUDY DESIGN AND COHORT CHARACTERISTICS**

### 3.1 Introduction

#### 3.1.1 The Republic of The Gambia

The Republic of The Gambia is the smallest country in mainland Africa with a population of 1,883,051 million ((CIA), 2013) and an annual population growth rate of 2.9% down from 4.2% during the 1983 to 1993 inter-census period (Department of Central Statistics, 2003). The country is narrow and takes its shape from the River Gambia with a total land area of 11,295 sq km; 10,000 sq km of which is dry land and 1,295 sq km is covered by water. It is less than 48.2 km at its widest end (the mouth of the Atlantic Ocean). The Gambia has borders with only one country, Senegal, which surrounds it on three sides and has 50 miles of coastline on the Atlantic Ocean on the west ((CIA), 2013).



**Figure 3.1** Map of the Gambia and location of MRC (UK), Gambia Unit and MRC Sukuta field site.

There are 5 major tribes in The Gambia: Mandinka (42%), Fula (18%), Wolof (16%), Jola (10%), Sarahule (9%), others 4% and non-African 1% (Department of Central Statistics, 2003). The population comprises approximately 90% Muslims, 9% Christians, with the remaining 1% following indigenous beliefs (US. Department of State, 2007).

**3.1.2 Public Health - Infant Immunizations**

Life expectancy in The Gambia for males and females is 57 years and 60 years respectively. The neonatal mortality rate is 31 per 1,000 live births; <1 year mortality is 57 per 1,000 live births; and under five mortality is 98 per 1,000 live births ((WHO)-UNICEF, 2011). Five years after The WHO established The Expanded Programme of Immunization (EPI) in 1974, The government of The Gambia implemented the National EPI program in 1979 which currently offers free vaccination against major diseases such as tuberculosis (BCG), diphtheria, tetanus and pertussis (DTwP), measles, polio and yellow fever, hepatitis B (HBV), *Haemophilus influenza* type B (Hib) and *Streptococcus pneumonia* (13-valent conjugated pneumococcal vaccine PCV-13) (see Table 1.1, Chapter 1). At the time of doing this study the EPI scheme was slightly different to the current one as shown in Table 3.1.

**Table 3.1 The EPI schedule at the time of the study, indicating vaccines given and blood samples taken throughout the study period**

| Age   | Vaccine Groups    |                   |                   |
|-------|-------------------|-------------------|-------------------|
|       | MV Group          | MV+DTwP Group     | DTwP Group        |
| Birth | OPV/BCG/HBV       | OPV/BCG/HBV       | OPV/BCG/HBV       |
| 1m    | OPV               | OPV               | OPV               |
| 2m    | DTwP1/Hib/OPV/HBV | DTwP1/Hib/OPV/HBV | DTwP1/Hib/OPV/HBV |
| 3m    | DTwP2/Hib/OPV     | DTwP2/Hib/OPV     | DTwP2/Hib/OPV     |
| 4m    | DTwP3/Hib/OPV/HBV | Hib/OPV/HBV       | Hib/OPV/HBV       |
| 6m    | VitA              | VitA              | VitA              |
| 9m    | Blood sample      | Blood sample      | Blood sample      |
| 9m    | MV                | MV/DTwP3          | DTwP3             |
| 10m   | Blood sample      | Blood sample      | Blood sample      |
| 11m   | YF/OPV            | YF/OPV            | MV/YF/OPV         |

**Key**

|      |  |
|------|--|
| OPV  | Oral polio vaccine   |
| BCG  | Bacillus Calmette Guerin                                       |
| HBV  | Hepatitis B virus  |
| DTP  | Diphtheria, tetanus with whole-cell pertussis combined vaccine |
| Hib  | Haemophilus influenza B  |
| VitA | Vitamin A  |
| MV   | Measles Vaccine  |
| YF   | Yellow Fever   |

*The key vaccine differences are in blue text and the age at which the study intervention occurred are shaded in light purple. All outstanding vaccines were administered at 11 months of age (yellow fever and OPV for the MV group and the MV+DTP group, and MV, OPV and YF for the DTP group).*

It is estimated that 2.5 million child deaths could be prevented each year by vaccination with notably higher survival rates in children who received all scheduled vaccines by 9 months of age. Vaccination therefore is central in the aim to reduce infant mortality and to achieve millennium development goal 4 (MDG4) – the reduction of under-five mortality rates by two thirds by 2015 (World Health Organization (WHO), 2005; World Health Organization (WHO), 2009). A study in The Gambia to assess vaccine coverage and the determinants of immunization in a semi-rural area in The Gambia reported high national coverage rates, 73% for measles vaccine, 86% for BCG and 92% for HBV1, but reduced

coverage in older children (>1 year) (Payne et al., 2013). WHO estimated 95% coverage for many immunizations (measles, yellow fever and third dose of DTP, Hib, HBV and polio) among 1-year-olds in The Gambia in 2009 ((WHO)-UNICEF, 2011).

### **3.1.3 MRC Sukuta Field Site**

The Sukuta Health Centre is in a peri-urban area in Kombo district, 20 km from the main MRC Laboratories in Fajara. It is a Government Health facility and has been collaborating with MRC for more than a decade. The MRC clinic was established in January 2001 as a field site for clinical trials and provides an excellent setting to examine the development of the infant immune system in response to vaccination. Sukuta is a stable community, which is very important for follow-up of the children enrolled in studies. The field site consists of a team of field workers, qualified nurses and 2 paediatricians. The field workers are provided with motorbikes to enable them to visit study participants in their homes during follow-up in all seasons. We also have a study database developer and a data entry team that double enters and verifies all data collected. All samples collected from the field site are transported to MRC Fajara within an hour for processing by the infant immunology laboratory team who have extensive experience in handling and processing small volume blood samples from infants.

## **3.2 Study design**

### **3.2.1 Ethical approval and informed consent**

The Joint Gambia Government/MRC Ethics Committee, and the London School of Hygiene and Tropical Medicine Ethics Committee approved the study protocol. A subject information sheet was given to parents/guardians that were considering joining the study. For those that could not read, it was read to them in their native language and the field worker ensured that the information was well understood. Those who agreed were asked to sign or thumbprint the consent form. The subject information sheet and the consent form are attached as appendix 1 and 2 respectively at the end of the thesis. The study

was conducted according to International Conference of Harmonization/ Good Clinical Practice (ICH-GCP) guidelines.

### **3.2.2 Vaccination study groups, follow up and bleeding schedule**

The results described in this thesis are part of a larger study (SCC1085) on understanding the broad immunological effects of infant measles or DTwP vaccination, or a combination of both vaccines. I led the laboratory aspects of the entire study. Infants presenting for their first dose of routine DTwP vaccine at 2 months of age at Sukuta Health Centre in The Gambia were identified and the parents/guardians approached; the study was explained and they were invited to participate in the study. The selection criteria required that all vaccines be up-to-date for these infants according to the current EPI schedule (Table. 3.1), and that children were healthy and afebrile on the day that they were recruited. Sick and underweight infants were therefore excluded. Informed consent was obtained from the parent/guardian at this 2 month visit. At 4 months of age the infants were randomized into one of three vaccine groups (Table 3.1) by picking an envelope containing the vaccine group (envelopes were arranged in sequential order and randomization performed in blocks by an independent statistician). Males and females were randomised separately to allow stratification by sex.

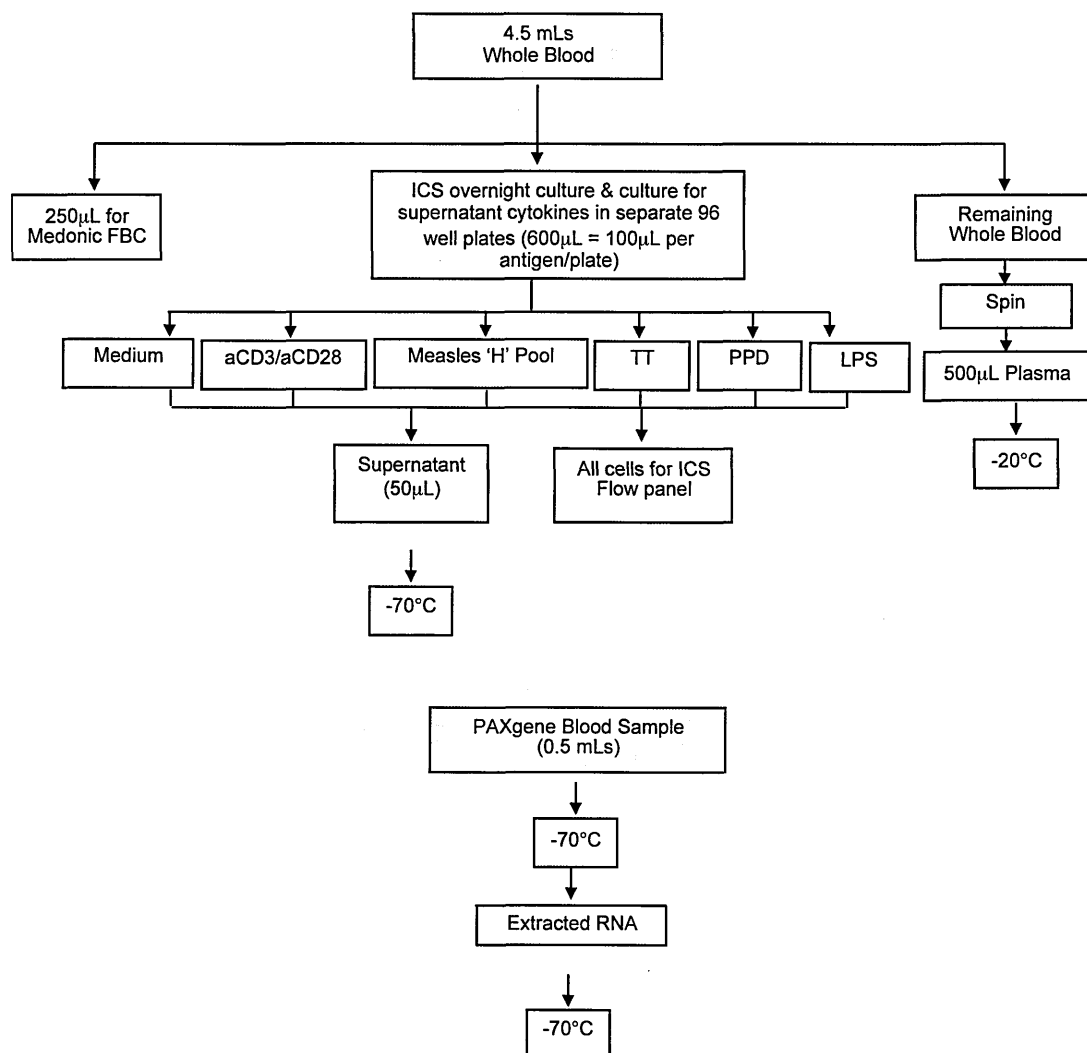
The randomized infants received different vaccine schedules at 4, 9 and 11 months of age according to which study group was randomly selected: Group 1 (MV group) received the normal EPI schedule of DTwP3, HBV and OPV at 4 months, whereas Group 2 (MV+DTwP group) and Group 3 (DTwP group) donors had their DTwP3 dose withheld and only had HBV and OPV (Table 3.1) above. At 9 months of age the MV group children received a single intramuscular (im) dose of measles vaccine (Edmonston Zagreb, Serum Institute of India Ltd, Pune, India) into the right deltoid; the MV+DTP group received MV in the left deltoid and an im dose of DTwP (UNICEF) in the thigh; and the DTwP group received DTwP alone as for MV+DTwP group. Any child that dropped out of the study was

given any missed vaccines according to the recommended EPI schedule and a drop out form was completed.

### **3.2.3 Blood handling**

4.5 mls of venous blood was collected from the 9-month old study participants prior to vaccination into a heparinized tube (7.5 units heparin per ml of blood) and at 4 weeks post-vaccination (10 months old); and 0.5 mls of whole blood was also collected into a Paxgene tube (Qiagen) for later RNA extraction and whole genome microarray transcriptome analysis at both time points. All samples were immediately transported to MRC Fajara for processing or storage as appropriate (Figure 3.2). MRC field staff recorded the vaccines given and these were then entered into a database and a schedule of follow up was set up to maintain the appropriate vaccine schedules for each child.

On arrival at the laboratory, 250µl of blood was used for full blood count (FBC) analysis on a CA620-20 Balder parameter system (Boule Medonic, Stockholm, Sweden). 1.2 ml of whole blood was used (100µl/well) for setting up the cultures in two separate plates for overnight intracellular staining (ICS) and for analysis of cytokines in culture supernatants by Bio-plex assay, as indicated in the flowchart (Figure 3.2). The remaining blood was spun and plasma collected and stored at -20°C until required for use for the vaccine antibody assays, and PBMC separated and stored in liquid nitrogen for future use. PAXgene blood samples were stored at -70°C for later RNA extraction in batches.



**Figure 3.2 Blood handling flowchart.** On arrival in the laboratory, the blood samples were divided between the assays to be performed as indicated in the flowchart.

### 3.2.4 Study Documentation

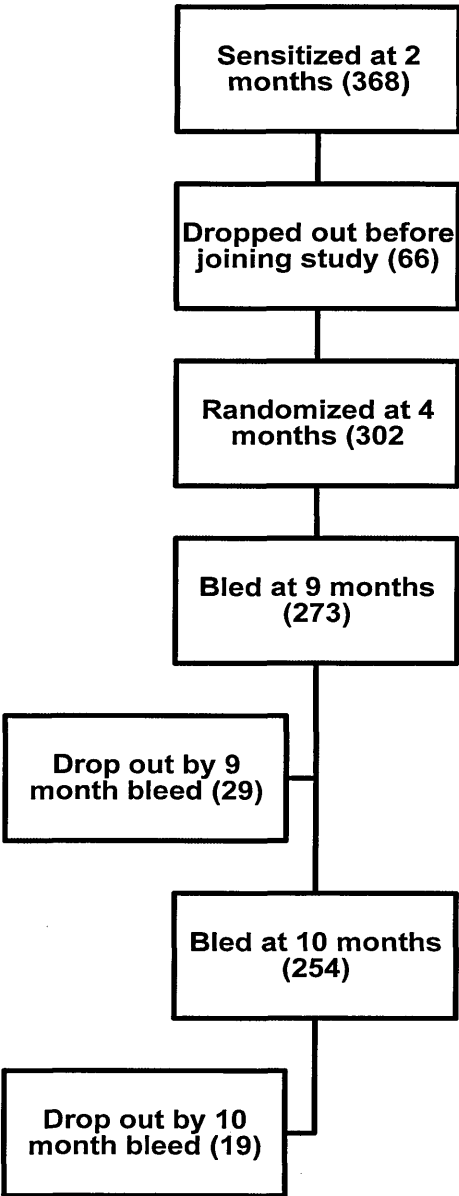
A series of case report forms (CRFs) were prepared for the study. At the first study visit information about the child's contact details and health were collected including a full vaccination history. Children were then followed up monthly from 2 months of age to 19 months, and CRFs completed including health details and vaccines given and recording those blood samples taken. If a child failed to attend for a scheduled visit the reasons were documented and efforts made to retain the child in the study. If a child dropped out of the study, a drop out form was completed and any missing vaccines were given. Separate forms documented the location of stored samples and results of the FBC from the Medonic analyzer. The data management team created a secure Microsoft Access database that held all the subject information.



**3.3 Study numbers & cohort characteristics**

**3.3.1 Study numbers**

A total of 368 infants were sensitized at 2 months of age in order to ensure adequate study numbers, and 302 of them were randomized into the study at 4 months of age. Blood samples were received from 273 infants at the 9 month time point and 254 at the 10 month time point. Thus, 29 infants (9.6%) dropped out of the study between 4 and 9 months, and a further 19 (6.3%) more infants dropped out by the 10 month bleed (Figure 3.3). Of these approximately half were males and half females since males and females were randomized separately.



**Figure 3.3 Hierarchy flow chart:** demonstrating the subjects retained and lost from the study at each time point.

3.3.2 Study Dropouts

There were a total of 48 study participants that dropped out of the study for various reasons by 10 months, accounting for 16% of the 302 recruited into this study, which is less than the predicted 20% dropout rate. This rate is similar to previous longitudinal studies carried out in Sukuta. Any child that deviated from the study protocol was removed from the study and a drop out form completed. Table 3.2 provides details of the reasons for participant drop out.

Table 3.2 No of drop outs and reason for dropping out of the study

| Reason for Drop out    | 9 month | 10 month |
|------------------------|---------|----------|
| Withdrew consent       | 13      | 9        |
| Deviated from Protocol | 2       | 1        |
| Travelled              | 7       | 4        |
| Died                   | 1*      | 0        |
| Relocated              | 6       | 5        |
| Total                  | 29      | 19       |

*\* Subject died after recruitment and before receiving any vaccine  
The number of participants that dropped out after recruitment and the reasons for doing so. Those that travelled dropped out because they missed a bleed and their time of return was not known.*

3.3.3 Cohort Characteristics

There was no difference in ethnicity between the groups with the majority being Mandinka, while Wolof and Fula constituted between 2 - 3% and 2 - 6% in the three vaccine groups respectively (Table 3.3). The median weight of the male children was slightly higher at baseline compared to females in all 3 groups (Table 3.3). All females and males infants in the study were being breastfed at baseline in the three groups. Formula milk feeding was not common in the three groups. These variables are therefore unlikely to be confounders in this study as the numbers are similar in female and male infants in the three vaccine groups.

**Table 3.3 Cohort characteristics for the three vaccine groups**

| Characteristics                               | MV group        |                 | MV+DTwP group   |                 | DTwP group      |               |
|---|-----------------|-----------------|-----------------|-----------------|-----------------|---------------|
|   | Female          | Male            | Female          | Male            | Female          | Male          |
| <b>No. in Study</b>                           | 48              | 58              | 56              | 59              | 37              | 44            |
| <b>Ethnicity Mother (Father) %</b>            |                 |                 |                 |                 |                 |               |
| Mandinka                                      | 38 (31)         | 42 (34)         | 47 (47)         | 41 (36)         | 27 (23)         | 29 (28)       |
| Wolof   | 1 (5)           | 5 (4)           | 3 (4)           | 3 (4)           | 2 (4)           | 3 (4)         |
| Fula  | 3 (5)           | 6 (10)          | 3 (3)           | 7 (11)          | 5 (4)           | 5 (5)         |
| Jola  | 3 (4)           | 2 (4)           | 0 (0)           | 3 (1)           | 1 (2)           | 3 (1)         |
| Other   | 3 (3)           | 3 (6)           | 3 (2)           | 5 (7)           | 2 (4)           | 4 (6)         |
| <b>Education Mother (years)</b>               |                 |                 |                 |                 |                 |               |
| <3 yrs  | 6               | 12              | 10              | 6               | 4               | 3             |
| 3-10 yrs                                      | 30              | 36              | 32              | 39              | 23              | 30            |
| >10 yrs                                       | 9               | 9               | 21              | 10              | 8               | 8             |
| Unknown                                       | 3               | 1               | 2               | 4               | 2               | 4             |
| <b>Breastfeeding status</b>                   |                 |                 |                 |                 |                 |               |
| Yes   | 48              | 57              | 56              | 59              | 37              | 44            |
| No  | 0               | 1               | 0               | 0               | 0               | 0             |
| <b>Formula (milk) feeding</b>                 |                 |                 |                 |                 |                 |               |
| No  | 31              | 36              | 31              | 31              | 23              | 29            |
| Yes   | 2               | 8               | 7               | 10              | 1               | 1             |
| Unknown                                       | 15              | 14              | 18              | 18              | 13              | 14            |
| <b>Other fluid</b>                            |                 |                 |                 |                 |                 |               |
| No  | 13              | 14              | 18              | 18              | 12              | 14            |
| Yes   | 34              | 43              | 36              | 38              | 25              | 30            |
| Unknown                                       | 1               | 1               | 2               | 3               | 0               | 0             |
| <b>Water</b>                                  |                 |                 |                 |                 |                 |               |
| No  | 1               | 3               | 1               | 1               | 0               | 0             |
| Yes   | 34              | 41              | 37              | 40              | 25              | 30            |
| Unknown                                       | 13              | 14              | 18              | 18              | 12              | 14            |
| <b>Weight of child ( Kg)</b><br>(Median, IQR) | 6.6 (6.1 - 7.2) | 7.3 (6.7 - 8.1) | 6.5 (5.9 - 7.2) | 7.1 (6.6 - 7.8) | 6.3 (5.9 - 6.8) | 7.3 (6.6 - 8) |
| <b>Lived in Sukuta (years)</b>                |                 |                 |                 |                 |                 |               |
| <2 yrs  | 1               | 0               | 4               | 3               | 3               | 3             |
| 2-10 yrs                                      | 10              | 12              | 16              | 18              | 7               | 13            |
| >10 yrs                                       | 12              | 10              | 9               | 14              | 10              | 14            |
| always  | 24              | 36              | 27              | 24              | 17              | 15            |
| Unknown                                       | 1               | 0               | 0               | 0               | 0               | 0             |

### 3.3.4 Full blood count results

A full blood count (FBC) was performed on all children at 9 and 10 months of age. No significant differences were found between any of the vaccine groups for any of the blood indices pre-vaccination when males and females were analyzed together. No sex differences were found for any of the parameters tested (Table 3.4).

Table 3.4 Summary of FBC results in males and females pre-vaccination (9 months)

|   | Pre-vaccination (9M)  |                       |                       |                       |                       |                      |
|---|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|----------------------|
|   | MV                    |                       | MV+DTwP               |                       | DTwP                  |                      |
|   | Median<br>(IQ range)  | Median<br>(IQ range)  | Median<br>(IQ range)  | Median<br>(IQ range)  | Median<br>(IQ range)  | Median<br>(IQ range) |
|   | M                     | F                     | M                     | F                     | M                     | F                    |
| Haemoglobin (Hb) g/dL                           | 10.2<br>(9.2 - 11.1)  | 10.2<br>(9.3 - 10.8)  | 9.8<br>(8.9 - 10.9)   | 9.9<br>(8.7 - 9.9)    | 10<br>(9.4 - 10.7)    | 10.1<br>(9.6 - 10.5) |
| White blood cell (WBC) x 10 <sup>3</sup> /µl    | 7.8<br>(5.3 - 9.8)    | 8.4<br>(7 - 10.6)     | 8.8<br>(6.7 - 12.1)   | 8.8<br>(7.3 - 11.4)   | 9.2<br>(6.3 - 10.2)   | 9.9<br>(7.1 - 11.1)  |
| Lymphocytes %                                   | 61.1<br>(55.8 - 67.8) | 63.4<br>(52.4 - 74.5) | 62.3<br>(60.5 - 66.4) | 64.8<br>(59.6 - 72.1) | 63.4<br>(50 - 69.1)   | 62.6<br>(56.3 - 69)  |
| Red blood cell (RBC) x 10 <sup>6</sup> /µl      | 5.2<br>(4.2 - 5.2)    | 4.7<br>(4.4 - 4.9)    | 4.7<br>(4.3 - 5.3)    | 4.5<br>(4.2 - 4.7)    | 4.6<br>(4.3 - 5.0)    | 4.5<br>(4.3 - 5.1)   |
| Packed cell volume (PCV) %                      | 32.6<br>(29.7 - 34.5) | 33.2<br>(29.8 - 34.7) | 31.9<br>(29.2 - 35.7) | 32.3<br>(29.1 - 33.8) | 31.9<br>(30.5 - 34.6) | 32.4<br>(31 - 34.5)  |
| Mean cell volume (MCV) fL                       | 68.9<br>(62.1 - 71.9) | 70.8<br>(67.5 - 73.4) | 68<br>(65.6 - 72.5)   | 71.9<br>(8.7 - 76.1)  | 68<br>(64.4 - 73.1)   | 71.9<br>(66 - 76.5)  |
| Mean cell Haemoglobin concentration (MCHC) g/dL | 31.1<br>(30.1 - 31.9) | 30.9<br>(30 - 31.4)   | 31.1<br>(29.8 - 32.3) | 31.2<br>(29.8 - 32.0) | 30.9<br>(30.3 - 31.6) | 31.3<br>(30 - 31.7)  |
| Platelets (x 10 <sup>9</sup> cells /L)          | 141<br>(103 - 251)    | 165<br>(104 - 265)    | 183<br>(121 - 267)    | 197<br>(135 - 272)    | 208<br>(122 - 263)    | 178<br>(144 - 265)   |

*Median and Interquartile range (IQR) of blood indices at baseline (9 months) in males and females in the three vaccine groups.*

3.3.5 Co-infections

We did not assess the HIV status of the infants, but based on the UNICEF 2011 statistics for Gambia, seroprevalence among adults was 1.5% and 0.1% among children (0-14 years of age) (UNICEF, 2005). This implies that very few children were likely to be HIV positive considering the sample size of this study. Low levels of helminth infections have been reported in other studies conducted near to Sukuta, with levels ranging from 0-3% in the adult population (Finney et al., 2009)and there is no schistosomiasis or filariasis in the area; it is therefore also unlikely that helminth infection would be a significant confounder in this study.

Overall the characteristics of this study cohort were very similar to previous infant studies carried out in Sukuta (Miles et al., 2008; Burl et al., 2010; Flanagan et al., 2010), indicating that our cohort is a true representation of the Sukuta community.

## **CHAPTER 4**

**VACCINE ANTIBODY TITRES ARE NOT AFFECTED BY COMBINING MV WITH DTWP  
OR BY THE SEX OF THE INFANT**

## 4.1 Introduction

Generally, the mainstay of vaccine research and development has focused on the induction of protective antibodies. B cells are produced in the bone marrow and are essential for production of antibodies to induce long-lasting protection after vaccination. Apart from fighting infections, CD4+ T cells, in particular Th2 cells, are also vital for providing help to B cells for antibody production (Reviewed in (Plotkin, 2001). Upon first encounter of infants to an antigenic agent, production of immunoglobulin M (IgM) results, but this later undergoes class switching to the isotypes IgG and IgA, which are more protective (Reviewed in (Achkar and Ziegenbalg, 2012).

IgG generated *in utero* generally wanes after the first 6 months of life (Achkar and Ziegenbalg, 2012), with only 30% of infants still having an appreciable level of circulating maternal antibodies. The titre of maternal antibodies (MAb) present at the time of immunization is thought to be the main determinant of the MAb-mediated inhibition of antibody responses in both mice and humans (Markowitz et al., 1996; Gans et al., 1998; Siegrist et al., 1998a; Siegrist et al., 1998b; Gans et al., 1999). In order to overcome this, EPI vaccines are either given early in multiples e.g. DTwP vaccine at 8, 12 and 16 weeks of age; or the vaccine is given after maternal antibody levels have waned e.g. the first dose of measles vaccine is given at 9 months of age in The Gambia.

Measurement of antibodies is crucial in determining the induction of protection against many infectious diseases. The terminology “protective antibody titres” usually refers to protection under usual conditions of exposure, with an average challenge dose and in the absence of inhibiting host factors (Plotkin, 2001). A study in rural Senegal reported a high degree of protection against measles infection in immunized and unimmunized children with neutralizing measles antibody titres >125 mIU (Samb et al., 1995). Antitoxin antibodies can prevent diphtheria and tetanus infections. Partial protection is achieved between 0.01 and 0.1 IU of antibodies with complete protection achieved above this concentration (Reviewed

in (Plotkin, 2001)). Protective titres for pertussis have not yet been established, but it has been shown that pertussis toxoid on its own can confer protection (Trollfors et al., 1995).

Human studies examining sex-based differences in vaccine antibody responses are few but antibody responses to viral and bacterial vaccines have consistently revealed higher titres in females than males (Cook, 2008; Klein et al., 2010). Geometric mean titres of maternal measles antibodies were found to be significantly higher in girls at 9 months of age than boys ( $P=0.007$ ) (Martins et al., 2009). In another study the prevalence of serum IgG antibodies against measles, mumps, and rubella was greatly increased in girls than in boys who received the MMR vaccine at 12–15 months after birth (Dominguez et al., 2006); and others reported significantly higher antibodies to standard titre measles vaccine in young female adults than males (Green et al., 1994). Adult females have also been shown to have higher antibody titres in response to the combined hepatitis A and B virus vaccine than males (Van der Wielen et al., 2006; Höhler et al., 2007); and females aged 18 – 40 years had significantly higher neutralizing antibody titers to smallpox vaccine than males (Kennedy et al., 2009). In this chapter we describe the role of sex and vaccine schedule on vaccine-induced antibody levels in Gambian infants.

## **4.2 Aims**

1. To determine whether co-administering MV with the third dose of DTwP will alter the vaccine-specific EPI antibody levels compared to giving MV or DTwP alone.
2. To determine whether there are any sex differences in EPI antibody levels in the different vaccine groups, and whether a particular gender was more susceptible to the effects of combining the vaccines.

4.3 Methods

4.3.1 Donor samples

Plasma was obtained from centrifuged heparinized whole blood samples (9 month baseline and 4 weeks after vaccination) for measurement of EPI antibody titres as described in Methods (Chapter 2). The total number of samples used for the measurement of EPI antibody titres and the number of samples assayed for males and females are indicated in Table 4.1 below.

Table 4.1 Number of samples assayed for vaccine antibodies at each time point

|                    | MV group |       |         | MV+DTwP group |         | DTwP group |         |
|--------------------|----------|-------|---------|---------------|---------|------------|---------|
|                    | Total    | Males | Females | Males         | Females | Males      | Females |
| MV Abs 9 months    | 260      | 48    | 41      | 53            | 53      | 33         | 32      |
| MV Abs 10 months   | 254      | 51    | 39      | 49            | 49      | 33         | 33      |
| DTwP Abs 9 months  | 260      | 47    | 40      | 55            | 53      | 33         | 32      |
| DTwP Abs 10 months | 256      | 50    | 40      | 51            | 49      | 33         | 33      |

*Total number of samples analyzed for measles Abs by HAI, and DTwP antibodies by multiplex assay by group and sex. MV Abs refers to measles antibodies and DTwP Abs refers to Diphtheria, Tetanus and Pertussis antibodies.*

4.3.2 Diphtheria, tetanus and pertussis antibodies by multiplex

The diphtheria toxoid (DTx), tetanus toxoid (TTx) and pertussis toxoid (PTx) multiplex antibody assays were carried out by our collaborators at The National Institute for Public Health and The Environment (RIVM), Bilthoven, The Netherlands as described in Methods (Chapter 2). Full protective levels for DTx and TTx were defined as greater than or equal to 0.1 IU/ml while partial protection is achieved at a titre of 0.01 - 0.09 IU/ml (van Gageldonk et al., 2011). A correlate of protection has yet to be established for pertussis, but PTx protective titres were defined by the lower limit of detection (LLOD) of each analyte as determined by interpolation of the +2 SD in the assay from the relevant reference curve. From the LLOD the lower limit of quantitation (LLOQ) was calculated as 3 x LLOD (van Gageldonk et al., 2008). An LLOQ of 1 Endotoxin Units (EU)/ml was applied for this study. Although there is no established correlate of protection for pertussis, a titre of ≥20 (EU)/ml is





often used in literature (de Greeff et al., 2010). The protective titre for PTx for this study was thus defined as  $\geq 20$  EU/ml.

It is worth remembering that all children had received DTwP at 8 and 12 weeks of age according to the EPI schedule, and the MV group also received their 3<sup>rd</sup> dose at 16 weeks of age. The MV+DTwP and DTwP groups had their third dose delayed until 9 months of age. Thus all children were expected to have detectable DTP antibody levels at the baseline bleed at 9 months of age.

#### **4.3.3 Measles (HAI) antibody assay**

The measles haemagglutinin inhibition (HAI) antibody assay was carried out at the Medical Research Council (MRC) Laboratories in The Gambia by Jainaba Njie-Jobe and Lady Chilel Sanyang as described in Methods (Chapter 2). The minimum detection level is 3.12 IU, and a protective measles antibody titre by HAI is defined as  $\geq 12.5$  IU antibody or a  $\log_2$  titre  $\geq 3$ , as described (Samb et al., 1995). Measles HAI antibody titres have been found to correlate with neutralizing antibody titres (Samb et al., 1995), and the assay is therefore considered a functional assay.

#### **4.3.4 Statistical analysis**

Differences in antibody levels between the 3 vaccine groups pre- and post-vaccination were analysed using a Kruskal-Wallis test followed by Dunn's post test comparison. Mann-Whitney U-tests were used to determine differences between pre- and post-vaccination antibody levels for each group and between males and females within each group. A  $p$ -value  $\leq 0.05$  was considered significant and are indicated by: \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$  and \*\*\*\* =  $p < 0.0001$ . *P-values  $< 0.035$  were considered significant to account for FDR*

## 4.4 Results

### 4.4.1 No effect of administering MV and DTwP together on vaccine-specific antibody responses compared to giving either vaccine alone

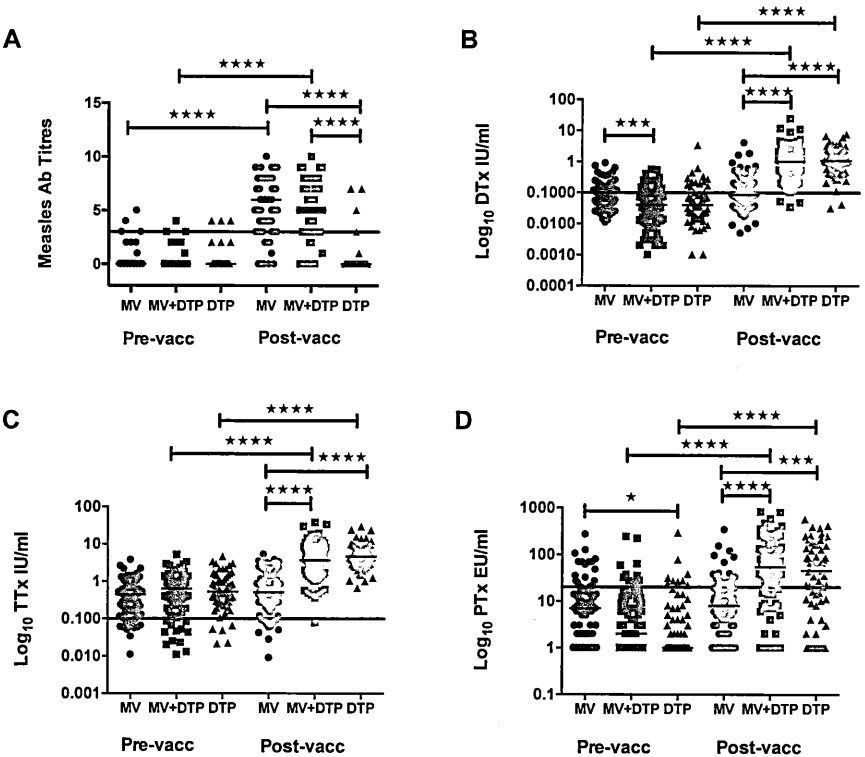
There were no group differences in measles titres pre-vaccination. A handful of subjects had low level pre-existing measles antibody titres at 9 months in all three vaccine groups which would be of maternal origin since none had received measles vaccine, and there had been no circulating measles infections in the area for some years (Figure 4.1A). Most of the pre-existing measles Ab levels were below the protective level of 3.0 suggesting that waning had occurred by 9 months of age. As expected the MV group and MV+DTwP groups had significantly higher antibody titres than the DTwP group post-vaccination since both the former received measles vaccine at 9 months whereas the DTwP group did not (Figure 4.1A). There was no difference in measles Ab titres when DTwP was administered with MV (MV+DTwP group) compared to when MV was given alone (MV group) (Figure 4.1A). Therefore giving measles vaccine alone or in combination with DTwP induced similar antibody titres in these children (Figure 4.1A). However, 30% of infants (36/133 male and 41/121 female) failed to achieve protective levels post vaccination in the MV group and MV+DTwP group. Interestingly, the majority of these infants did not have pre-existing maternal antibodies at 9 months except for two female infants in the DTwP group suggesting maternal antibodies are important for generating protective levels after vaccination.

Prior to Measles vaccination DTx antibody titres were significantly higher in the MV group than the MV+DTwP group (Figure 4.1B), which is expected since the former had received 3 doses of DTwP, whereas the MV+DTwP and DTwP groups had only received two doses. Post-vaccination, the MV+DTwP and DTwP groups boosted to equivalent levels, which were higher than the MV alone group, since they did not receive a DTwP booster. Almost all boosted infants achieved protective DTx titres (Figure 4.1B).

There were no vaccine group differences in TTx antibody titres pre-vaccination, even for the MV group who had received one more dose of DTwP than the MV+DTwP and DTwP

groups. As expected, the MV+DTwP and DTwP groups significantly boosted their TTx antibody levels post-vaccination but not group1 who did not receive a dose of DTwP (Figure 4.1C). 99% of boosted infants achieved protective TTx Ab levels, and the levels were comparable whether DTwP was given with MV or not.

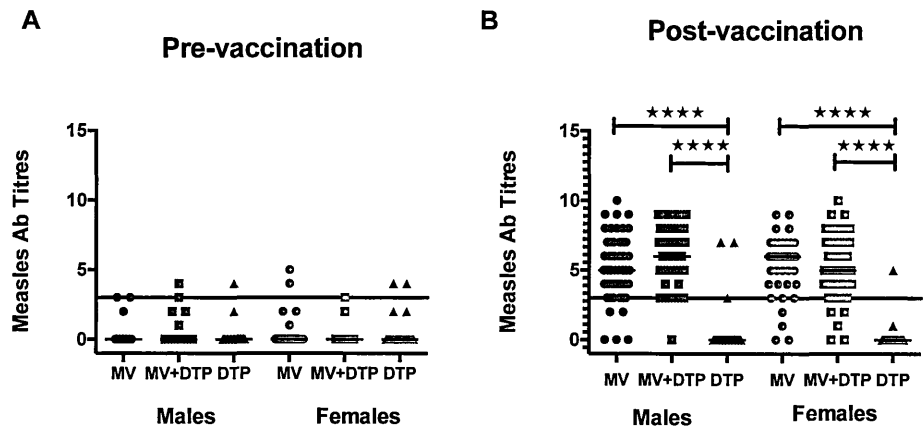
Pertussis antibody titres pre-vaccination were significantly higher in the MV group than the DTwP group after correction for multiple testing. As expected, at 10 months, the MV+DTwP and DTwP groups had significantly higher PTx antibody levels than the MV group (Figure 4.1D); the majority of the infants in the MV+DTwP and DTwP group boosted above protective levels, although a significant number failed to suggesting that the protective cutoff may be an overestimate. Again, no difference in PTx Abs was observed if MV was delivered at the same time (MV+DTwP group) compared to alone (DTwP group) (Figure 4.1D).



**Figure 4.1 EPI antibody titres.** MV (Gp1), MV+DTwP (Gp2) and DTwP (Gp3) antibody titres to measles (A), diphtheria toxoid (DTx) (B), tetanus toxoid (TTx) (C) and pertussis toxoid (PTx) (D) pre-vaccination and post-vaccination in the 3 study groups. Titres pre-vaccination are indicated by blue dots, and post-vaccination by red dots. The median is indicated by the horizontal line. The horizontal line across all groups indicates the protective titre for that particular antibody. The Kruskal-Wallis test and Dunn's post-test comparison was used to compare antibody levels between the three vaccine groups. Adjusted P-values are shown as stars at the top of the graph, \* indicates P < 0.05, \*\*\* indicates P < 0.001 and \*\*\*\* indicates P < 0.0001.

4.4.2 Antibody responses are comparable in males and females

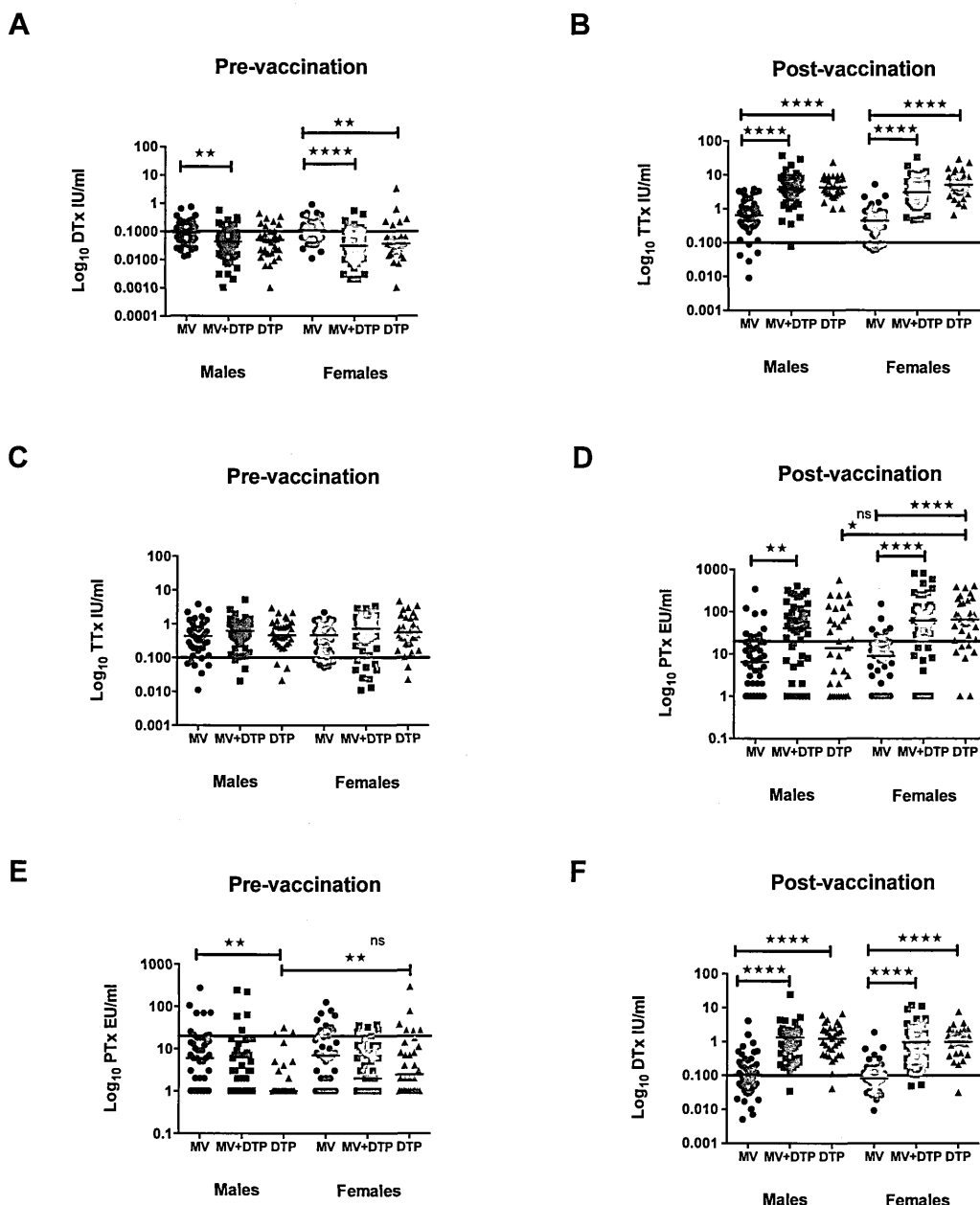
When analysed by sex, no differences were seen in pre- or post-vaccination measles antibody titres within the same vaccine group (Figure 4.2A). After vaccination both males and females boosted their measles titres in the MV groups and MV+DTwP group as expected (Figure 4.2B).



**Figure 4.2 Measles antibody titres by sex.**

Measles-specific antibody titres in the 3 study group male and female vaccinees pre-vaccination at 9 months (A) and post-vaccination at 10 months (B). Antibody titres in male subjects are indicated by blue dots, and females by red dots. The median is indicated by the horizontal line. Measles-specific antibody titres are expressed log<sub>2</sub> titre. Protective levels are considered to be a titre of  $\geq 3.0$ . Horizontal line across all groups indicates the protective titre of that particular antibody. The Kruskal-Wallis test and Dunn's post-test comparison was used to compare antibody levels between the three vaccine groups. Adjusted P-values are shown as stars at the top of the graph. \*\*\* indicates  $P < 0.0001$ .

Males and females had equivalent Ab titres to DTx and TTx pre- and post-vaccination at each vaccine group (Figure 4.3A and C) and the expected boost occurred in the MV+DTwP and DTwP vaccinated groups (Figure 4.3B and D). By contrast, there were sex differences in PTx antibody levels both pre- (Figure 4.3E) and post-vaccination (Figure 4.3F). The DTwP vaccinated females had higher PTx Abs than males both pre-vaccination ( $P=0.0053$ ) and post-vaccination ( $P=0.0104$ ). The expected post-boost increase occurred in the MV+DTwP and DTwP groups.



**Figure 4.3 DTP antibody titres by sex.**

Diphtheria toxoid (DTx), tetanus toxoid (TTx) and pertussis toxoid (PTx) antibody titres in MV, MV+DTP and DTP male and female vaccinees pre-vaccination (A, C and E) and post-vaccination (B, D and F). Antibody titres in male subjects are indicated by blue dots, and females by red dots. The median is indicated by the horizontal line. DTx, TTx PTx-specific antibody titres are expressed on a logarithmic ( $\log_{10}$ ) scale. Protective levels for DTx and TTx are considered to be  $\geq 0.1$ , and for PTx  $\geq 20$  Endotoxin Units (EU)/ml. Horizontal line across all groups indicates the protective titre of that particular antibody. The Kruskal-Wallis test and Dunn's post-test comparison was used to compare antibody levels between the three vaccine groups. Adjusted P-values are shown as stars at the top of the graph, \* indicates  $P < 0.05$ , \*\* indicates  $P < 0.001$  and \*\*\* indicates  $P < 0.0001$ , ns indicates P-values were not significant after Dunn's post test comparison.

## 4.5 Discussion

Overall, our results suggest that combining MV with DTwP has no effect on vaccine antibody titres compared to when either vaccine is given alone. The booster dose of DTwP3 administered at 9 months to the MV+DTwP group and the DTwP group infants produced a significant recall antibody response four weeks after vaccination, with almost all infants achieving protective levels. This demonstrates that a booster dose of DTwP at 9 months of age does improve antibody levels. Even though no antibody correlate of protection has yet been established for pertussis, it is reassuring that pertussis antibody titres were comparable in subjects vaccinated with measles plus DTwP and those vaccinated with DTwP alone, with both groups boosting their pre-vaccination level. In a study in which children were vaccinated at 3, 4, and 5 months of age with DTwP vaccine, the authors reported adequate protection against diphtheria, tetanus and pertussis until the age of preschool booster (Ramsay et al., 1991).

Thirty percent of measles vaccinated infants failed to achieve protective measles antibody titres 4 weeks after vaccination. This was not due to pre-existing maternal antibodies, as only two infants with pre-existing maternal antibodies were amongst those that failed to achieve protective levels. It may partly be attributed to the fact that these infants were measles naïve and this was the first dose administered to them.

There were no sex differences in measles, diphtheria toxoid or tetanus toxoid antibody titres in the MV, MV+DTwP or DTwP groups after vaccination. There was a significantly lower PTx titre in males in the DTwP group at baseline, which is difficult to understand since they should have behaved similarly to the MV+DTwP group at baseline since both had received 2 doses of DTwP. The low baseline titre probably accounted for the same male / female difference observed post-vaccination, albeit less pronounced than at baseline. However it does fit with the paradigm that females generally have higher vaccine Ab levels than males, as described in the introduction. The high number of infants with PTx titres below protective levels four weeks after vaccination in the MV+DTwP and the DTwP groups suggests that

the protective titre may be set too high given that these infants are thought to be protected against pertussis infection. Overall however, our data do not support clear male female differences in Ab titres to measles or DTwP vaccines but do show that combining vaccines is not detrimental to inducing protective antibody titres to any of the study-specific vaccines we used.



**CHAPTER 5**

**ANALYSIS OF SOLUBLE CYTOKINE PROFILES IN INFANTS IN RESPONSE TO  
VACCINE-SPECIFIC AND NON-SPECIFIC ANTIGENS DEPENDING ON  
THEIR VACCINATION STATUS AND SEX**

## 5.1 Introduction

In Chapter 4, vaccine group or sex did not affect vaccine antibody titres. We next wanted to determine if there was an influence on innate and adaptive responses by analysing the secreted cytokine pattern after culture with vaccine specific and un-related antigens in the three vaccine groups. The primary aim was to determine if there were differences for sex and vaccine group as well as non-specific effects of the vaccines administered by using non-vaccine related antigens. There are many reports regarding induction of vaccine-specific T cell responses and immune polarization (ie towards a pro- or anti-inflammatory response) following administration of measles vaccine (MV) or diphtheria, tetanus, whole cell pertussis combined vaccine (DTwP) to animals and humans. However little is known about their broader, non-specific immunological effects. Live vaccines such as measles vaccine (MV) are generally thought to cause a T helper type 1 (Th1) skewed immune response to measles antigens, while killed vaccines such as the combined vaccine diphtheria, tetanus, whole cell pertussis (DTwP) are thought to skew towards Th2 type immune responses to the vaccine components (Ward and Griffin, 1993). However, while MV has been shown to induce a strong pro-inflammatory IFN- $\gamma$  response in humans (Pauksen et al., 1997), the Th1-polarising cytokine IL-12 had also been shown to be down-regulated by MV, leading to the hypothesis that MV ultimately skews T cells towards Th2 (anti-inflammatory) immune responses (Karp et al., 1996).

Despite the fact that such immune skewing by vaccines is well described, there is insufficient information regarding the consequences in humans in terms of disease patterns and outcomes. It is not known whether such immune polarizing effects apply to responses to non-vaccine antigens, but if they do one would predict that they would influence the outcome of infections. This is of particular relevance in countries, such as The Gambia, with a high level of endemic infections.

The cytokine milieu in which dendritic cells process and present antigen to naïve T cells is very important in determining polarization into different T cell subsets, and can also cause

certain populations of memory cells to change their phenotype. The outcome of the immune response is dependent on the predominant cytokine secretion pattern. The Th1/Th2 focus of previous studies does not take into account other key arms of the immune system such as innate responses, which inevitably influence adaptive immunity, regulatory cytokines, and the Th17 pro-inflammatory pathway.

The non-specific effects (NSE) of vaccines have been found to be particularly prominent in females in epidemiological studies (Aaby et al., 1995; Benn and Aaby, 2012). The mechanisms accounting for these sex-differences are not known, but it has been suggested that X-linked immune response genes, miRNAs and sex hormones might have a role (Flanagan et al., 2011). Few immunological or vaccine studies have analyzed males and females separately, thus there are limited data on sex differences in vaccine-induced immunity in humans. Understanding the cytokine secretion patterns to non-vaccine antigens might identify key sex differences that account for the observed sex differences in NSE and mortality.

A major aim of this chapter was to investigate whether the different vaccine schedules cause a skewing of the immune profile in response to *in vitro* stimulation with vaccine specific and non-vaccine antigens. We hypothesized that differences would also be associated with the sex of the infant.

### Specific Aims

1. To evaluate the *in vitro* cytokine profiles to vaccine and non-vaccine antigens of infants vaccinated with MV alone, DTwP alone or MV+DTwP administered at the same time.
2. To determine whether males and females differ in their cytokine responses following the different vaccine regimens.
3. To determine immune profiles that might account for beneficial / deleterious NSE of MV and DTwP respectively that can be tested in future prospective studies.

## 5.2 Methods

### 5.2.1 Antigen stimulation

100µl of heparinized whole blood from infants was stimulated for 16 hours with vaccine-specific and non-vaccine antigens. All available blood samples from 9 month (pre-vaccination baseline, n = 169) and 10 month (4 weeks post-vaccination, n = 170) old infants were analysed. As detailed in Chapter 3, all infants had received their routine EPI vaccines up to 12 weeks of age, and all had received at least two doses of DTwP. The conditions tested *in vitro* were:

1. Tetanus toxoid (TT), 10 µg/ml (to determine the DTP-specific response)
2. Measles haemagglutinin 20-mer peptide pool, 1 µg/ml for each peptide (to test for MV-specific response)
3. Purified protein derivative (PPD) of *Mycobacterium tuberculosis*, 10 µg/ml (to test for reactivity to an unrelated recall antigen). All infants had received the BCG vaccine in the first week of life.
4. Anti-CD3/anti-CD28, each at 5 µg/ml (as a global T cell stimulus (positive control))
5. Unstimulated i.e. 100µl of whole blood without antigen stimulation (negative control for spontaneous cytokine production)

### 5.2.2 Multiplex cytokine assay

Following overnight stimulation, supernatants were collected and assayed using the Bioplex assay protocol (Bio-Rad Laboratories, France), as described in section 2.2.2 of Methods, for the cytokines detailed in Table 5.1. An initial 27-plex assay was performed on selected samples as described in Methods and the 10 most informative cytokines which differentiated vaccine groups were selected for analysis of all remaining samples.

**Table 5.1 Cytokines selected for the 10-plex assays**

| Polarity   | Analyte   |
|------------|---|
| Th1        | IFN- $\gamma$ , IL-12(p70), TNF- $\alpha$                     |
| Th2        | IL-4, IL-10   |
| Regulatory | IL-10   |
| Innate     | IL-1 $\beta$ , TNF- $\alpha$ , GM-CSF, PDGF-BB, VEGF, Eotaxin |

*Cytokines selected for the 10-plex assay were interferon gamma (IFN- $\gamma$ ), interleukin-12(p70), interleukin-4, interleukin-10, interleukin-1 $\beta$ , tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), granulocyte macrophage-colony stimulating factor (GM-CSF), platelet-derived growth factor-BB (PDGF-BB), vascular endothelial growth factor (VEGF) and eotaxin.*

Spider plots were generated for easy visualization of cytokine data using Microsoft Excel® for Mac 2011, Version 14.2.2. Log<sub>10</sub> of each cytokine median value was plotted around a vertical axis creating a global view of the changes in cytokine profile in the three vaccine groups. All median values less than 1 were first set to 1 to give a log<sub>10</sub> value of 0.

**5.2.3 Generalized Least-Squares (GLS) Regression Analysis**

Background (unstimulated) cytokine production was subtracted prior to analysis of antigen-specific cytokine levels. Cytokine production in the unstimulated cultures was also analyzed, as this is informative for the underlying cytokine milieu in the different vaccine groups. STATA 12.1, Copyright 1985-2011 StataCorp LP, Statacorp USA, was used for all analyses (<http://www.stata.com>). The data were ranked and fitted to a Generalized Least-Squares (GLS) regression model to compare differences between groups and between males and females within each group at each time point, which seemed the most appropriate approach for analysing the non-parametrically distributed cytokine data. The GLS approach uses parametric methods of analysis for which a number of non-normal distributions can be specified. After writing and fitting the model, data checks were performed and normalization done so that the data becomes approximately normal in distribution. The model was used for each condition and all our analysis was derived from it. We compared cytokine levels of the three vaccine groups at 9 months and at 10

months. We also assessed whether being male or female (effect of sex) affected cytokine levels in a group. To study sex differences, we directly compared males and females within each group at 9 months and at 10 months. False Discovery Rate (henceforth referred to as FDR) was applied for p-value adjustment (Benjamini and Hochberg, 2005). The FDR accounts for multiple adjustments when more than 1 parameter is being measured in an assay. It controls the false discovery rate, that is, the expected proportion of false positives among the variables for which there is a significant difference. After calculation, an unadjusted p-value of  $\leq 0.035$  was taken as significant to account for the FDR and are indicated.

### **5.3 Results**

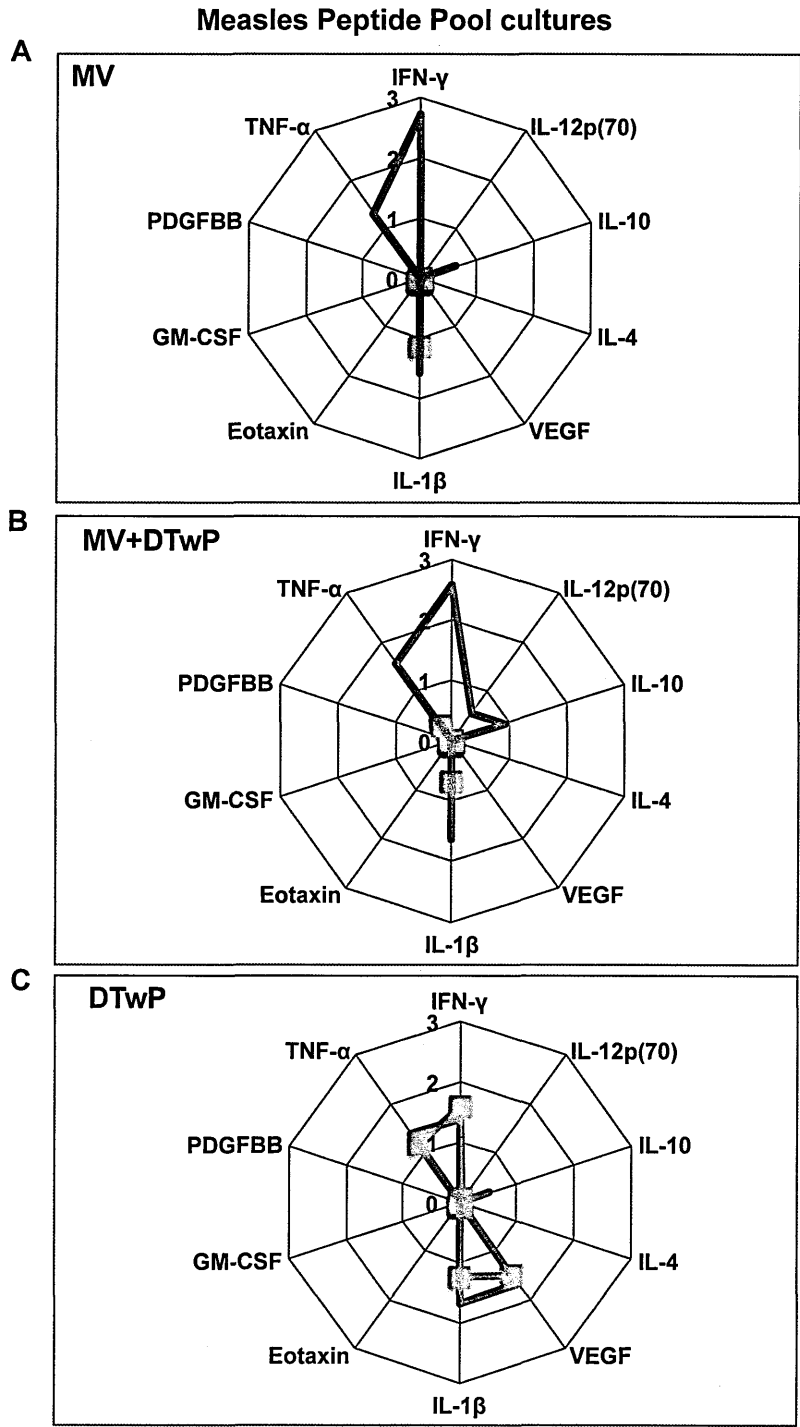
#### **5.3.1 Responses to positive and negative control antigens were unaffected by vaccine group when all infants were analysed together**

In the unstimulated samples, there were no differences in any cytokines regardless of the stimuli, the vaccine group or the time-point. Similarly, when all donors are analysed together, stimulation with the  $\alpha$ CD3/ $\alpha$ CD28 positive control showed no significant differences between the three vaccine groups pre-or post-vaccination for any of the 10 cytokines tested (Tables 5.2, 5.3 and 5.4).

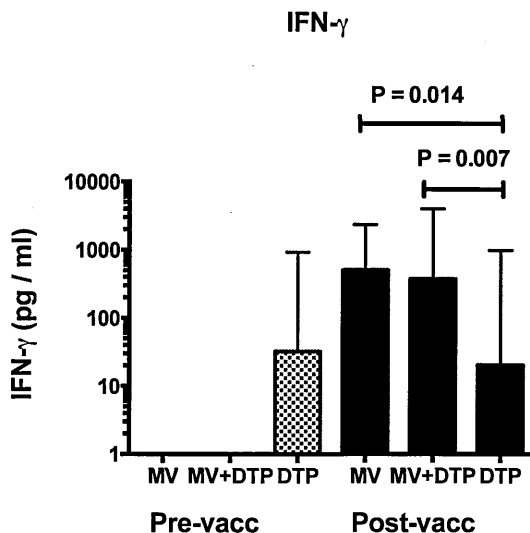
#### **5.3.2 Differences in vaccine-specific responses (MV Pool and TT cultures)**

As expected, following measles peptide pool cultures there was significantly higher IFN- $\gamma$  production in infants who received MV or MV+DTwP compared to those vaccinated with DTwP alone ( $p=0.014$  and  $0.007$  respectively) (Figures 5.1 and 5.2; Table 5.4). This type 1 cytokine bias is expected since only these 2 groups received MV 4 weeks earlier, and supports induction of an early measles-specific IFN- $\gamma$  response following MV administration. There was no induction of IL-4 or IL-10 in MV pool cultures post-vaccination, and VEGF, PDGFBB, GM-CSF, Eotaxin and IL-12(p70) levels were negligible in measles pool cultures (Figures 5.1A-C; Table 5.3). It is apparent from the spider plots

that IFN- $\gamma$  is the main cytokine induced by measles vaccination, compared to baseline responses at 9 months when infants were MV naïve (Figure 5.1).



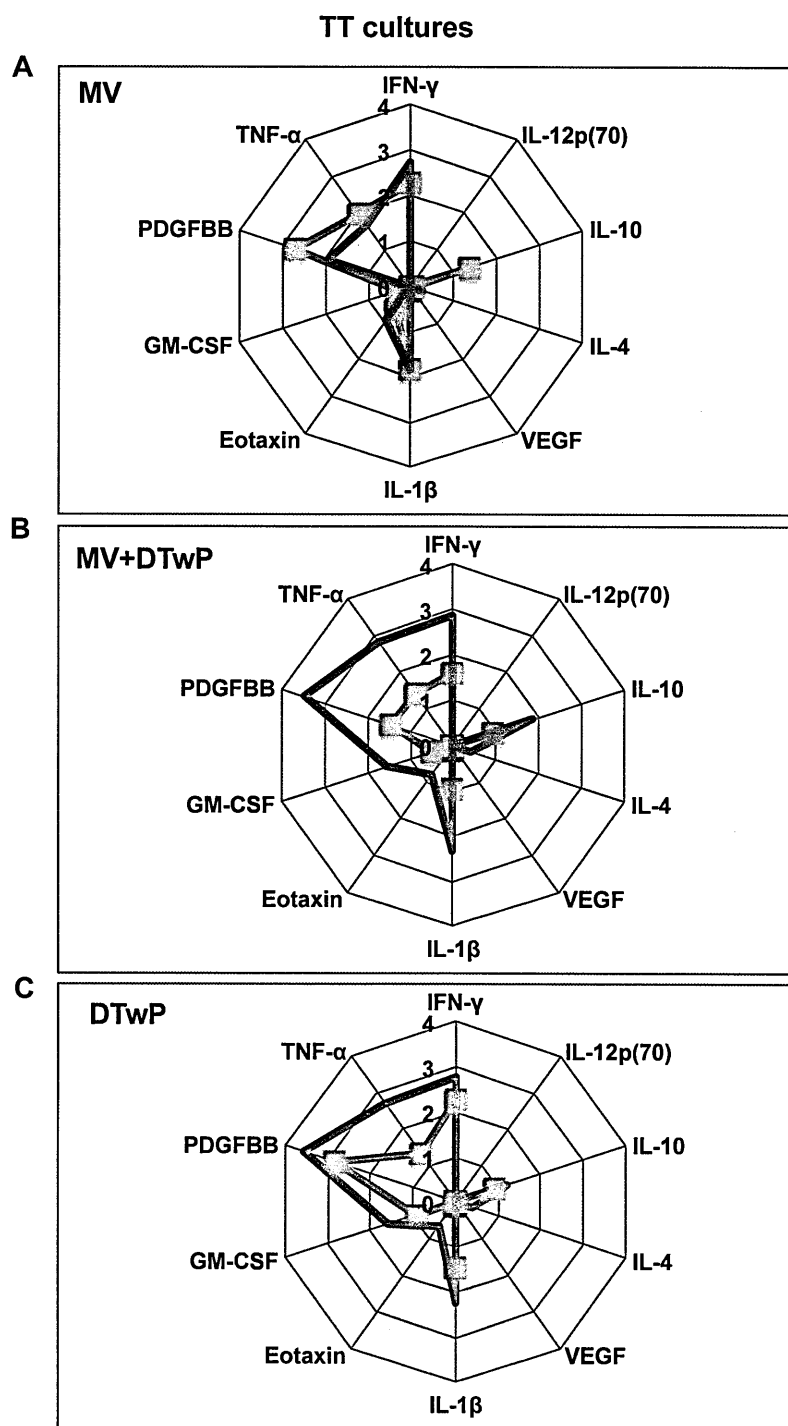
**Figure 5.1 Spider plots showing changes in cytokine responses in measles peptide pool cultures.** Log<sub>10</sub> of all median values were plotted in the spider plots around a vertical axis creating a global view of the changes in cytokine profile in the three vaccine groups. Blue line = pre-vaccination responses and purple line = post-vaccination responses. All median values <1 were set to 1 to give log<sub>10</sub> value of 0.



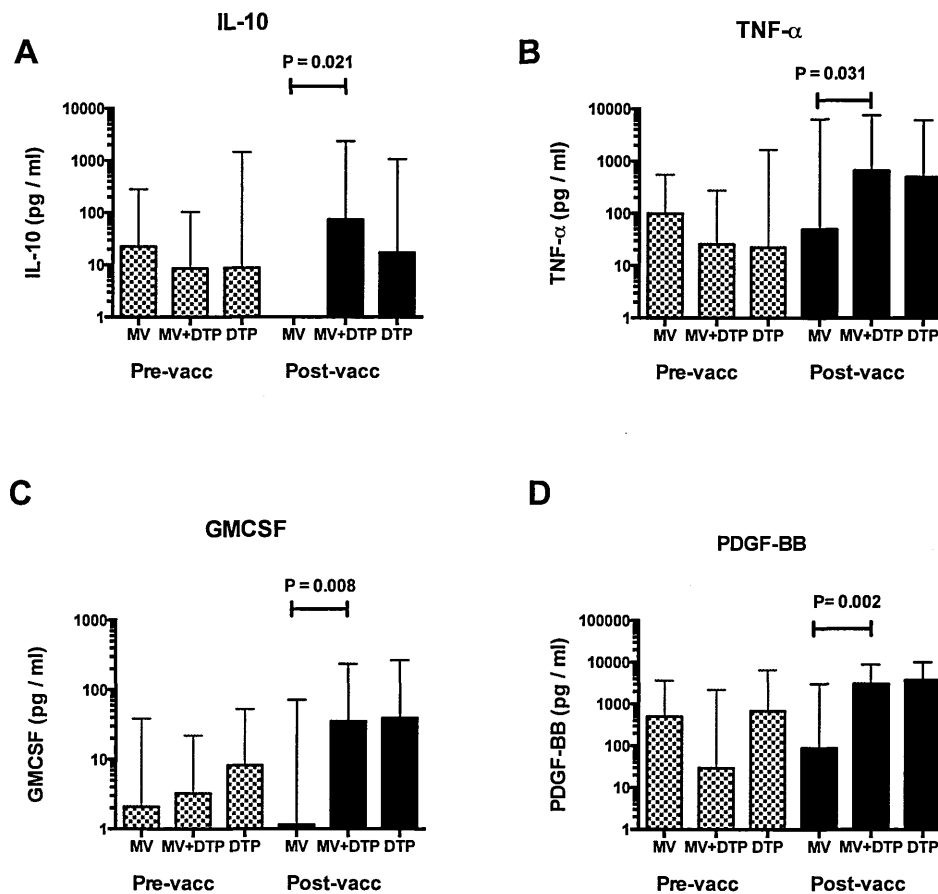
**Figure 5.2 IFN- $\gamma$  concentrations in supernatants following stimulation with measles peptide pool.** Median and interquartile range of IFN- $\gamma$  following background subtraction in the three vaccine groups (MV, MV+DTwP and DTwP) pre and post-vaccination. *P*-values were derived using GLS regression model and are indicated at the top of the bars. *P*-values <0.035 were considered significant to account for FDR and are indicated.

There was also a boosting of TT reactivity at 10 months in the MV+DTwP and DTwP as expected, but not the MV group who were not boosted (Figure 5.3). Stimulation with TT revealed significantly higher post-vaccination levels of IL-10, TNF- $\alpha$ , GM-CSF and PDGF-BB in the MV+DTwP group compared to the MV group who did not receive a booster (*P* values =0.021, 0.031, 0.008, 0.002 respectively) (Figures 5.3 and 5.4; Table 5.4). From the global view provided by the spider plots, the DTwP group seems to have boosted to similar levels to the MV+DTwP group (Figures 5.3B and C), but this group had higher baseline TT responses for IFN- $\gamma$ , PDGF-BB and IL-1 $\beta$ .





**Figure 5.3 Spider plots showing changes in cytokine responses in TT cultures.**  $\log_{10}$  of all median values were plotted in the spider plots around a vertical axis creating a global view of the changes in cytokine profile in the three vaccine groups. Blue line = pre-vaccination responses and purple line = post-vaccination responses. All median values  $<1$  were set to 1 to give  $\log_{10}$  value of 0.

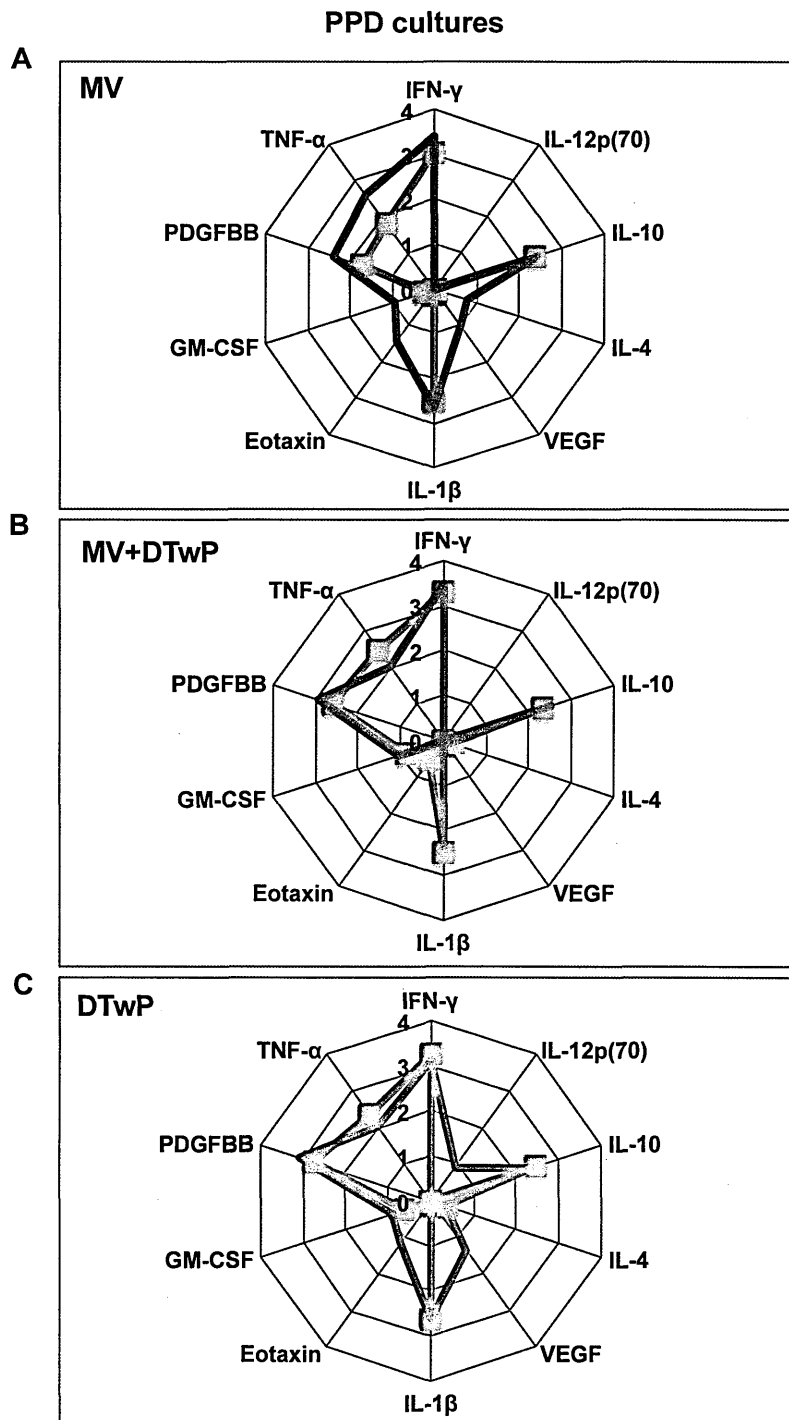


**Figure 5.4 IL-10, TNF- $\alpha$ , GM-CSF and PDGF-BB concentrations supernatants following TT stimulation.** Median and interquartile range of the cytokines following background subtraction in the three vaccine groups (MV, MV+DTwP and DTwP) pre- and post-vaccination. P-values were derived using GLS regression model and are indicated at the top of the bars. P-values  $<0.035$  were considered significant to account for FDR and are indicated.

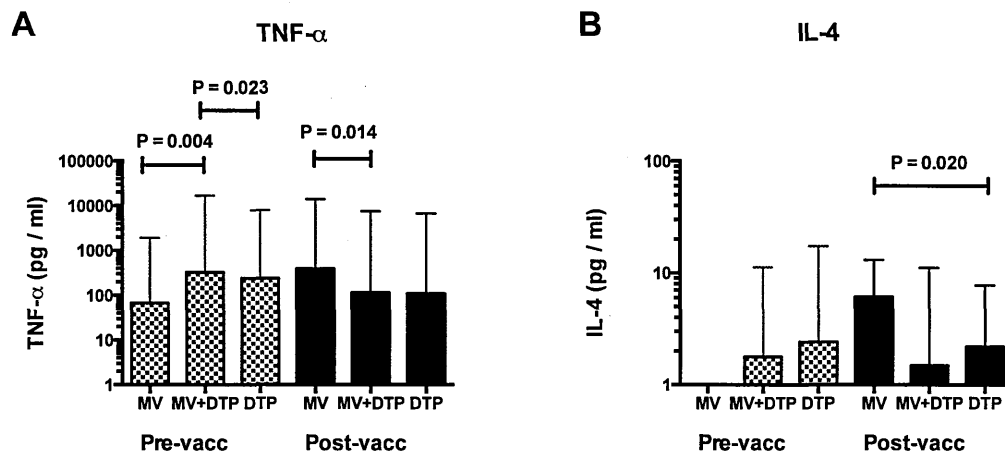
### 5.3.3 Responses to the non-vaccine antigen PPD are altered by vaccination group

BCG was routinely given to all children in the first week of life but was not one of the vaccines administered at 9 months as part of the study protocol. Thus any differential differential modification of PPD reactivity at 10 months of age (4 weeks after MV, DTwP or MV+DTwP vaccination) in the different vaccine groups would support an immunomodulatory non-specific or heterologous effect of vaccine administration. Following PPD stimulation, there were differences between groups both pre- and post-vaccination. The MV+DTwP group had higher baseline TNF- $\alpha$  production to PPD than the DTwP or MV groups pre-vaccination ( $P=0.023$  and  $0.004$  respectively). After vaccination it

was the MV group that had higher TNF responses than the MV+DTwP group suggesting enhanced reactivity to PPD in the MV infants (Figure 5.5A-C). There were also higher pre-vaccination levels of IL-12p(70) and VEGF in the MV+DTwP group compared to the MV group, although the median values were negative and thus the baseline difference was not likely of biological relevance (Figures 5.5A and B; Table 5.4). Production of IL-4 in PPD cultures was also significantly higher in the MV group than in the DTwP group post-vaccination ( $P=0.020$ ) (Figures 5.5A & C and 5.6B) (Tables 5.2 and 5.3).



**Figure 5.5 Spider plots showing changes in cytokine responses in PPD cultures.**  $\log_{10}$  of all median values were plotted in the spider plots around a vertical axis creating a global view of the changes in cytokine profile in the three vaccine groups. Blue line = pre-vaccination responses and purple line = post-vaccination responses. All median values  $<1$  were set to 1 to give  $\log_{10}$  value of 0.



**Figure 5.6** *TNF- $\alpha$  and IL-4 concentrations in supernatants following PPD stimulation.* Median and interquartile range of the cytokines following background subtraction in the three-vaccine groups (MV, MV+DTwP and DTwP) pre and post-vaccination. *P*-values were derived using GLS regression model and are indicated at the top of the bars. *P*-values <0.035 were considered significant to account for FDR and are indicated.

nterquartile ranges (IQ ranges) for all groups in the different stimulations pre-vaccination (9 months)

| MV Pool    |             |             |              | PPD          |              |             | TT           |              |             | aCD3/CD28   |             |              |
|------------|-------------|-------------|--------------|--------------|--------------|-------------|--------------|--------------|-------------|-------------|-------------|--------------|
| Median     |             |             |              | Median       |              |             | Median       |              |             | Median      |             |              |
| (IQ range) |             |             |              | (IQ range)   |              |             | (IQ range)   |              |             | (IQ range)  |             |              |
| Gp3        | Gp1         | Gp2         | Gp3          | Gp1          | Gp2          | Gp3         | Gp1          | Gp2          | Gp3         | Gp1         | Gp2         | Gp3          |
| 360        | -9          | -28         | 37           | 1068         | 1908         | 1854        | 200          | 40           | 178         | 1348        | 1319        | 2721         |
| (216-526)  | (-239-119)  | (-475-33)   | (-76-2169)   | (299-2623)   | (461-14385)  | (672-9801)  | (30-1207)    | (-131-707)   | (0-1915)    | (-52-7592)  | (-70-8150)  | (73-9907)    |
| 48         | 1           | 2           | 15           | 66           | 325          | 236         | 99           | 26           | 22          | 2313        | 4862        | 8198         |
| (7-130)    | (-21-46)    | (-23-55)    | (-2-1819)    | (3-1920)     | (20-15613)   | (0-7349)    | (3-523)      | (-24-277)    | (-1.4-1517) | (-2-18266)  | (2-18596)   | (25-25138)   |
| 6          | -1          | 0           | 0            | 1            | 2            | 2           | 2            | 0            | 1           | 6           | 8           | 11           |
| (3-12)     | (-3-2)      | (-2-1.6)    | (-2-17)      | (-0.6-9)     | (-1.2-11)    | (0-17)      | (-0.4-8)     | (-1-2)       | (-1-10)     | (-0.3-20)   | (-0.5-17)   | (1.4-25)     |
| 97         | -19         | -14         | -9           | -2           | 3            | -1          | 4            | -5           | 0           | 42          | 52          | 68           |
| (45-256)   | (-62-8)     | (-41-11)    | (-44-140)    | (-58-79)     | (-20-98)     | (-31-153)   | (-38-47)     | (-31-11)     | (-39-79)    | (-46-162)   | (-33-130)   | (-2.3-170)   |
| 42         | 0           | -2          | 0            | 243          | 217          | 299         | 23           | 9            | 9           | 2293        | 1982        | 2786         |
| (20-260)   | (-50-24)    | (-142-12)   | (-26-1005)   | (34-1224)    | (33-2406)    | (67-2737)   | (-5-261)     | (-33-104)    | (-16-1345)  | (14-3985)   | (72-4825)   | (422-7046)   |
| 23         | 14          | 5           | 17           | 331          | 320          | 444         | 67           | 9            | 29          | 2840        | 3279        | 5413         |
| (6-210)    | (-5-205)    | (-27-69)    | (-25-10277)  | (37-2341)    | (11-6661)    | (32-8360)   | (5-4108)     | (-37-166)    | (-29-6935)  | (2.7-8870)  | (39-8708)   | (1475-14374) |
| 97         | -1          | 0           | 1            | -12          | 0            | -3          | -16          | -20          | -9          | -4          | -1          | 19           |
| (65-153)   | (-22-44)    | (-30-45)    | (-24-118)    | (-57-11)     | (-29-148)    | (-36-58)    | (-59-14)     | (-20-3.2)    | (-35-19)    | (-41-27)    | (-31-57)    | (-41-95)     |
| 12         | 0           | 0           | 0            | 2            | 7            | 4           | 2            | 3            | 8           | 10          | 20          | 31           |
| (0.3-101)  | (-18-1.4)   | (-5-0.9)    | (-4-81)      | (0-19)       | (0-47)       | (0-47)      | (0-34)       | (-0.6-22)    | (0-40)      | (0-91)      | (0-59)      | (0-107)      |
| 5110       | -925        | -866        | -669         | 52           | 420          | 565         | 498          | 29           | 681         | 2591        | 1607        | 3776         |
| 2017-9425) | (-3277-522) | (-2938-676) | (-1367-1986) | (-1812-2775) | (-1051-4849) | (-780-6931) | (-1121-3647) | (-1349-2185) | (-963-6453) | (-917-6354) | (-684-6731) | (245-10940)  |
| 495        | -19         | -35         | 30           | -80          | -37          | -1          | -127         | -127         | -56         | -20         | 66          | 226          |
| (322-1120) | (-175-104)  | (-224-70)   | (-172-725)   | (-296-79)    | (-140-318)   | (-230-479)  | (-369-76)    | (-392-(-10)) | (-221-299)  | (-229-375)  | (-94-630)   | (-236-775)   |

values and interquartile ranges (IQR) for all cytokines analysed following all culture conditions.

Interquartile ranges (IQ ranges) for all groups in the different stimulations post-vaccination (10 months)

| MV Pool              |              |              |              | PPD                  |              |             |              | TT                   |             |              | aCD3/CD28            |              |
|----------------------|--------------|--------------|--------------|----------------------|--------------|-------------|--------------|----------------------|-------------|--------------|----------------------|--------------|
| Median<br>(IQ range) |              |              |              | Median<br>(IQ range) |              |             |              | Median<br>(IQ range) |             |              | Median<br>(IQ range) |              |
| Gp3                  | Gp1          | Gp2          | Gp3          | Gp1                  | Gp2          | Gp3         | Gp1          | Gp2                  | Gp3         | Gp1          | Gp2                  | Gp3          |
| 263                  | 501          | 369          | 23           | 2664                 | 2932         | 1240        | 561          | 736                  | 575         | 1701         | 416                  | 1072         |
| (148-1310)           | (-42-1516)   | (-80-3877)   | (-160-1034)  | (580-6506)           | (843-16272)  | (84-11623)  | (-281-2309)  | (70-3261)            | (17-1805)   | (-37-5778)   | (-496-6048)          | (-75-5980)   |
| 20                   | 21           | 37           | 26           | 384                  | 112          | 108         | 49           | 656                  | 497         | 7937         | 559                  | 3227         |
| (7-117)              | (-14-1595)   | (-8-6978)    | (-19-7741)   | (64-13874)           | (22-7126)    | (24-6694)   | (-12-5536)   | (17-7558)            | (22-5851)   | (4-27111)    | (-18-16432)          | (-13-14265)  |
| 4                    | 1            | 0            | 0            | 6                    | 2            | 2           | 2            | 3                    | 2           | 9            | 2                    | 7            |
| (2-9)                | (-3-11)      | (-2-10)      | (-3-9)       | (0.3-13)             | (-0.4-11)    | (-0.1-8)    | (-0.7-12)    | (-0.6-12)            | (0-8)       | (-0.4-19)    | (-5-14)              | (-0.3-13)    |
| 103                  | -15          | -15          | -7           | 27                   | -8           | 13          | 9            | 7                    | 4           | 57           | 5                    | 19           |
| (53-216)             | (-108-148)   | (-80-99)     | (-55-129)    | (-25-151)            | (-37-109)    | (-26-93)    | (-43-118)    | (-52-82)             | (-29-123)   | (-45-156)    | (-69-104)            | (-17-97)     |
| 49                   | 4            | 8            | 3            | 262                  | 342          | 236         | 0            | 73                   | 17          | 2063         | 1249                 | 1982         |
| (22-2513)            | (-26-676)    | (-43-1497)   | (-49-1131)   | (50-2143)            | (73-3261)    | (48-2124)   | (-57-586)    | (-8-2332)            | (-15-1059)  | (26-5662)    | (5-6701)             | (16-5226)    |
| 33                   | 38           | 43           | 48           | 441                  | 223          | 428         | 71           | 214                  | 171         | 2551         | 2263                 | 3042         |
| (8-302)              | (-1.33-8367) | (-50-3976)   | (-30-25928)  | (324-3885)           | (18-3164)    | (52-12367)  | (-4-2712)    | (0-4707)             | (129-11694) | (2-18453)    | (19-10433)           | (0.86-14738) |
| 83                   | 0            | 4            | 0            | -2                   | -2           | 8           | -3           | 0                    | -22         | -1           | 6                    | 21           |
| (32-127)             | (-31-38)     | (-12-35)     | (-25-72)     | (-31-12)             | (-32-28)     | (-18-35)    | (-31-11)     | (-36-16)             | (-51-26)    | (-42-23)     | (-31-50)             | (-18-76)     |
| 0                    | 0            | 0            | 0            | 9                    | 11           | 8           | 1            | 35                   | 39          | 29           | 6                    | 14           |
| (0.31-14)            | (-1.83-46)   | (-0.3-39)    | (-2.5-82)    | (0-53)               | (0-59)       | (0-55)      | (0-67)       | (0-230)              | (2-261)     | (0-158)      | (0-77)               | (0-81)       |
| 7146                 | -711         | -507         | -468         | 249                  | 851          | 1198        | 87           | 3033                 | 3732        | 1247         | 1252                 | 4666         |
| (-422-11703)         | (-2873-1045) | (-2870-2223) | (-1486-3561) | (-2370-3296)         | (-1532-3370) | (-303-5602) | (-1472-2924) | (-34-8497)           | (-991-9863) | (-1182-5812) | (-2612-4682)         | (-129-10191) |
| 483                  | 0            | 0            | 46           | 10                   | 1            | 24          | -17          | 0                    | -47         | 99           | 73                   | 211          |
| (207-930)            | (-127-251)   | (-259-329)   | (-140-412)   | (-274-257)           | (-188-199)   | (-115-353)  | (-223-112)   | (-274-303)           | (-305-117)  | (-213-408)   | (-180-484)           | (-101-513)   |

values and interquartile ranges (IQR) for all cytokines analysed following all culture conditions.

**Table 5.4 A summary of the interactions between groups**

| Stimulation      | Cytokine      | P-value | Responses      |
|------------------|---------------|---------|----------------|
| PRE-VACCINATION  |               |         |                |
| PPD              | IL-12p70      | 0.005   | MV+DTwP > MV   |
|                  | TNF- $\alpha$ | 0.004   | MV+DTwP > MV   |
|                  | VEGF          | 0.017   | MV+DTwP > MV   |
|                  | TNF- $\alpha$ | 0.023   | MV+DTwP > DTwP |
| POST-VACCINATION |               |         |                |
| Measles          | IFN- $\gamma$ | 0.007   | MV+DTwP > DTwP |
|                  | IFN- $\gamma$ | 0.014   | MV > DTwP      |
| PPD              | TNF- $\alpha$ | 0.014   | MV > MV+DTwP   |
|                  | IL-4          | 0.020   | MV > DTwP      |
| TT               | IL-10         | 0.021   | MV+DTwP > MV   |
|                  | TNF- $\alpha$ | 0.031   | MV+DTwP > MV   |
|                  | GMCSF         | 0.008   | MV+DTwP > MV   |
|                  | PDGFBB        | 0.002   | MV+DTwP > MV   |

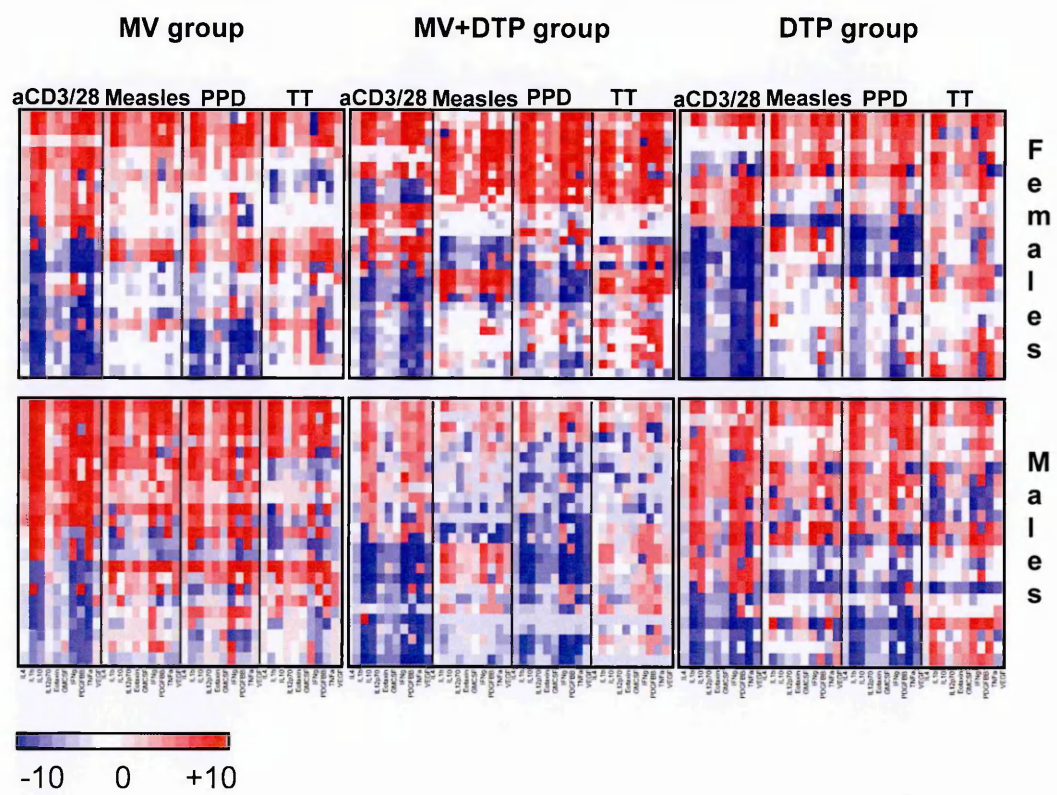
Only significant differences after FDR correction are shown in the table. PPD = purified protein derivative; Measles = measles peptide pool and TT = Tetanus toxoid. MV refers to the MV group; MV+DTwP refers to MV+DTwP group and DTwP refers to the DTwP group. >indicates responses that are higher in one group compared to the other, e.g. MV > DTwP = responses are significantly higher in the MV group than the DTwP group. P-values <0.035 were considered significant to account for FDR and are indicated.

### 5.3.4 Distinct cytokine pattern changes when the vaccine groups were analysed by sex

In order to get an idea of the overall pattern of cytokine changes by group and sex we calculated the fold-change post-vaccination in cytokine production following each stimulus for each cytokine compared to pre-vaccination levels. These were then plotted as heat-maps providing a global overview of the effect of vaccine group by sex (Figure 5.7). A few patterns were apparent using this approach. Firstly, the MV+DTwP females appeared to have more up-regulated (red) cytokines than MV females, whereas the MV+DTwP males seemed to down-regulate (blue) cytokines compared to the MV group males. The MV males had more up-regulated cytokines than MV females, and MV+DTwP females had more up-regulated cytokines than males. The MV group males had more up-regulated



cytokines than either the MV+DTwP or DTwP group, whereas for females it was the MV+DTwP group that had the most up-regulated profile after vaccination. Responses to the non-specific T cell stimulus  $\alpha$ CD3/ $\alpha$ CD28 and the unrelated antigen PPD also seem to vary according to vaccine group, supporting an influence of vaccination on reactivity to these antigens, and thus potential non-specific effects of vaccination on cellular immunity.

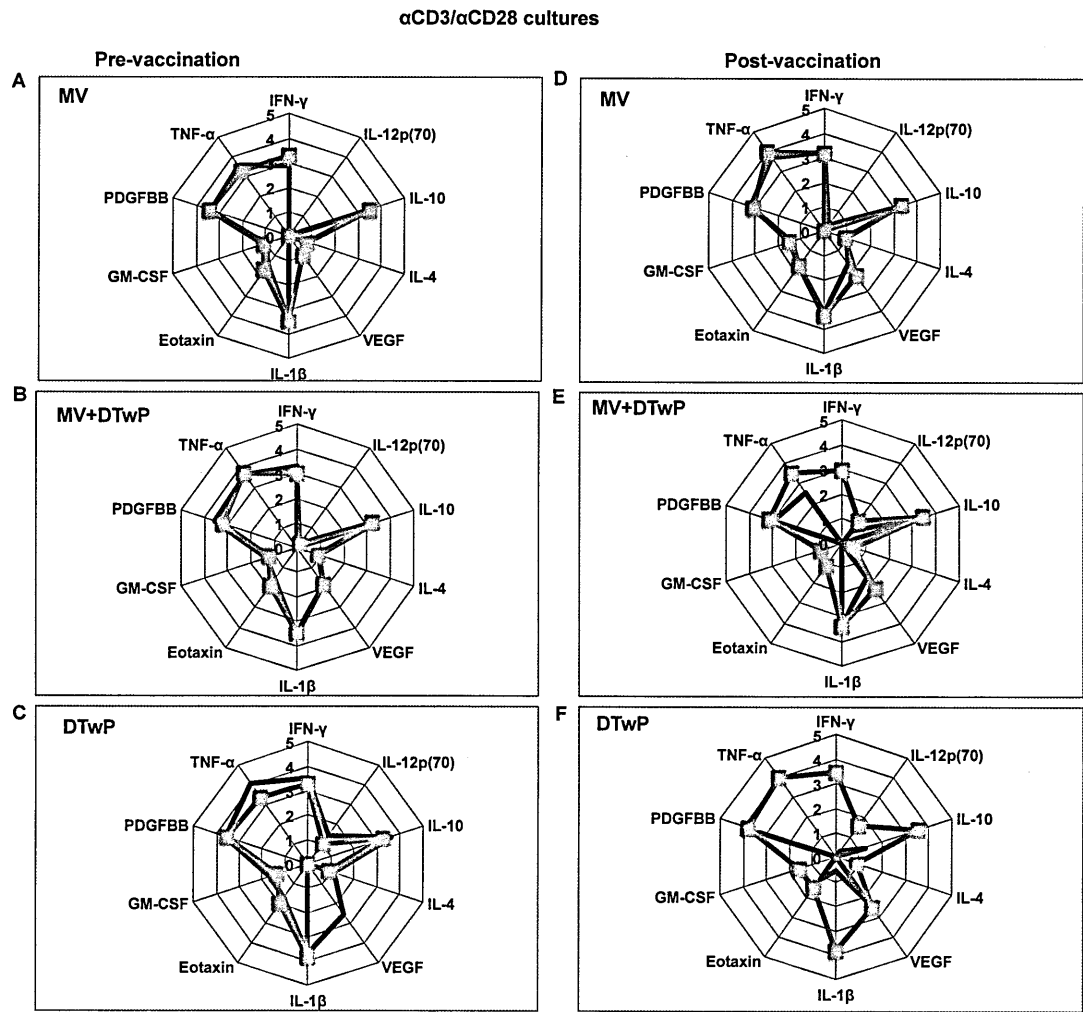


**Figure 5.7 Heatmaps of fold-change differences in cytokine concentrations.** Fold change differences in cytokine levels post-vaccination compared to pre-vaccination in females and males in the three vaccine groups. The 10 cytokines tested are indicated on the x-axis. Whole blood was stimulated overnight with  $\alpha$ CD3/28, measles pool, PPD or TT and supernatants analysed for levels of IL-4, IL1b, IL-10, IL-12(p70), Eotaxin, GMCSF, IFN- $\gamma$ , PDGFBB, TNF- $\alpha$ , VEGF. Red =increase, blue = decrease, white = no change post-vaccination compared to pre-vaccination (baseline).

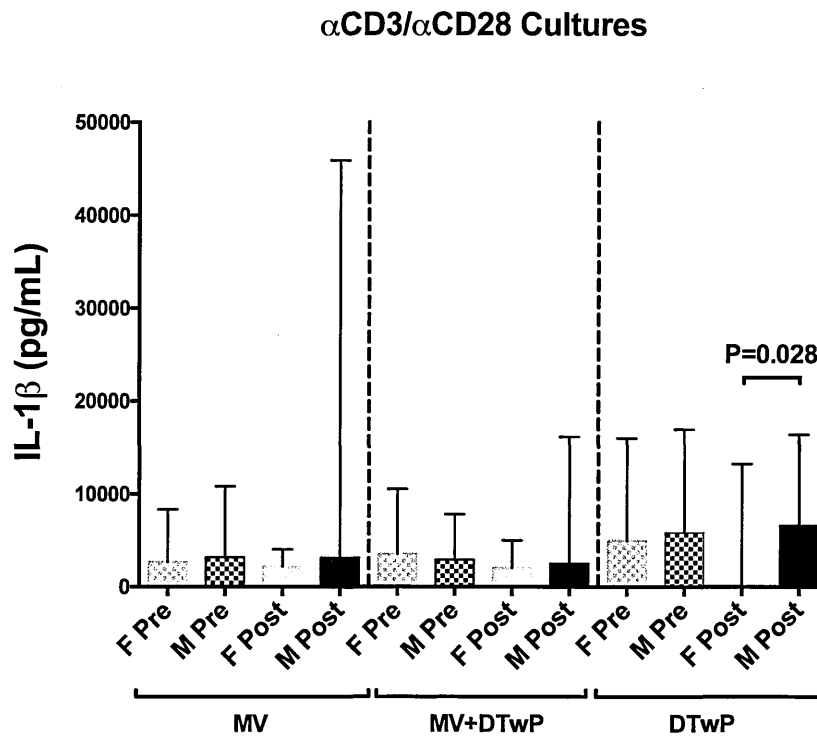
### 5.3.5 Cytokine down-regulation to $\alpha$ CD3/ $\alpha$ CD28 in females following DTwP vaccination

Interestingly, following  $\alpha$ CD3/ $\alpha$ CD28 stimulation, DTwP vaccinated females showed down-regulation of IL-1 $\beta$ , IFN- $\gamma$  and TNF- $\alpha$  production to a median value of almost zero suggesting a loss of inflammatory T cell function. However, only the reduced IL-1 $\beta$  was significantly lower in females in the DTwP group compared to males after adjusting for FDR (P=0.028, Figure 5.9, Table 5.5), while the IFN- $\gamma$  and TNF- $\alpha$  effects were not

significant. Such a down-regulatory effect was not observed in males (Figures 5.8F, 5.9C and D) and the spider plots reveal similar cytokine profiles in males and females pre-vaccination (Figure 5.8A-C).



**Figure 5.8 Spider plots showing changes in cytokine responses in  $\alpha$ CD3/ $\alpha$ CD28 cultures.** Log<sub>10</sub> of all median values were plotted in the spider plots around a vertical axis creating a global view of the changes in cytokine profile in the three vaccine group before and after vaccination by sex. Purple line = responses in females and blue line = responses in males pre (A-C) and post-vaccination (D-F). All median values <1 were set to 1 to give log<sub>10</sub> value of 0.



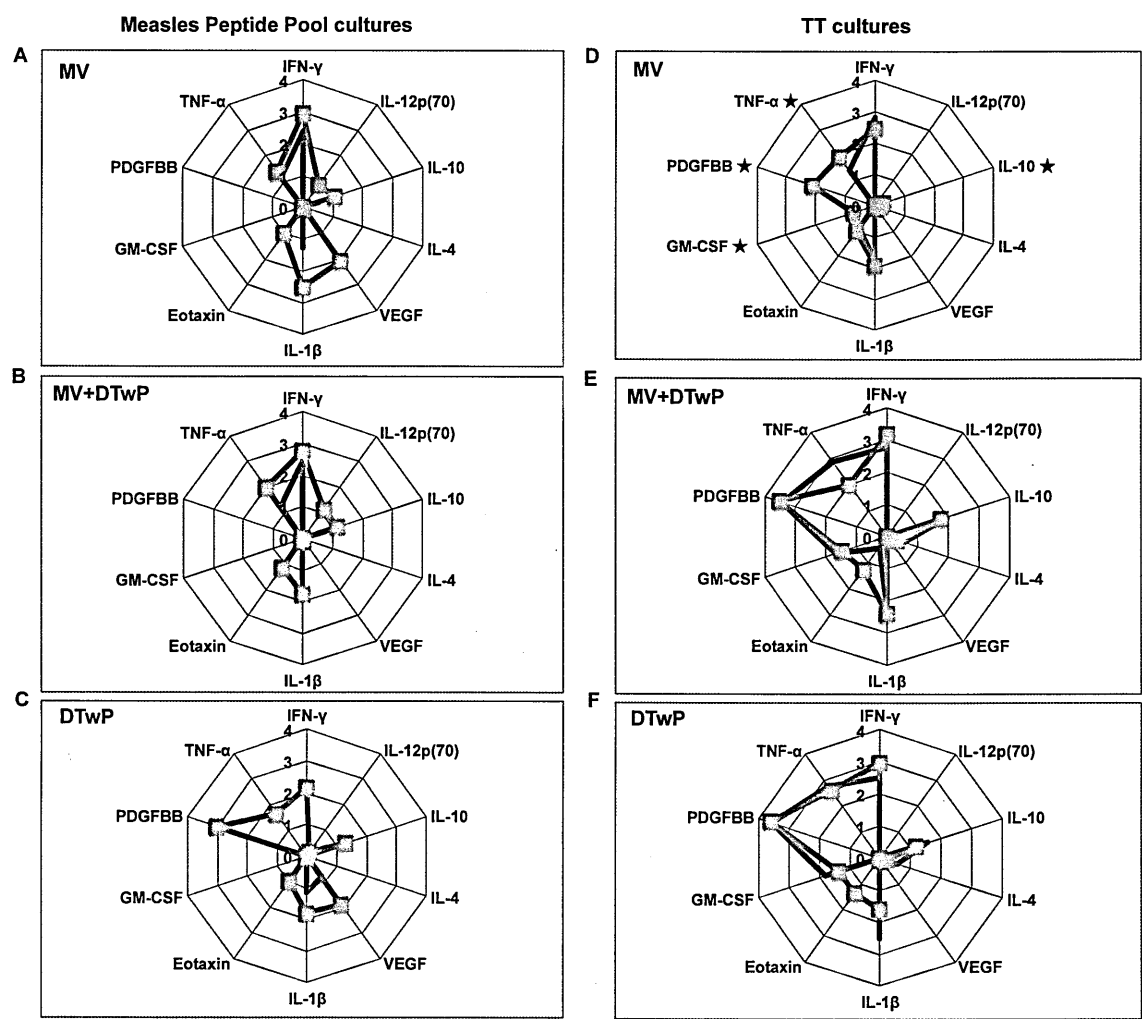
**Figure 5.9** Median cytokine levels in  $\alpha$ CD3/ $\alpha$ CD28 cultures for IL-1 $\beta$  in the 3 vaccine groups before and after vaccination in males and females. The error bar indicates the upper quartile. P-values were derived from the GLS model analysis and indicate the vaccine time point at which there was a significant effect of sex.

### 5.3.6 Sex differences in vaccine-specific responses following vaccination in the MV group

There were significant sex differences post-vaccination following stimulation with the vaccine-specific antigens. The MV males had higher IFN- $\gamma$  reactivity to MV Pool than MV females ( $P=0.031$ ; Figures 5.10A, 5.11B; Table 5.5). The MV group also had higher VEGF to measles antigen stimulation, than the MV+DTwP group who had almost no VEGF response (Figure 5.10A & B).

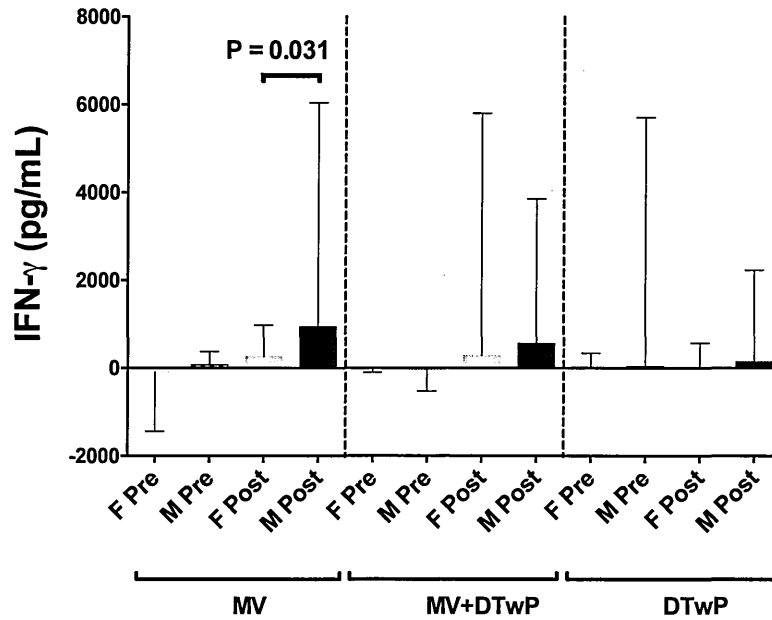
In the TT cultures, the MV group females had significantly lower TNF- $\alpha$ , GM-CSF, PDGF-BB and IL-10 production to TT than males post-vaccination ( $P = 0.030, 0.002, 0.003, 0.027$ , respectively), suggesting that MV enhanced reactivity in males (Figure 5.10D, Table 5.5). This male / female difference in TT reactivity was not observed when MV was

given with DTwP (Figure 5.10B), however this group had received a DTwP booster and therefore cannot be compared to the MV group. The spider plots demonstrate that males and females have comparable TT reactivity following a DTwP boost in both the MV+DTwP and DTwP groups (Figure 5.10 E and F).



**Figure 5.10 Spider plots showing changes in cytokine responses following stimulation with measles peptide pool and with TT.** Log<sub>10</sub> of all median values were plotted in the spider plots around a vertical axis creating a global view of the changes in cytokine profile in the three vaccine group before and after vaccination by sex. Purple line = responses in females and blue line = responses in males in measles peptide pool cultures (A-C) and TT cultures (D-F) post-vaccination. All median values <1 were set to 1 to give log<sub>10</sub> value of 0. A star next to a cytokine name indicates a significant difference between males and females.

## MV Pool cultures

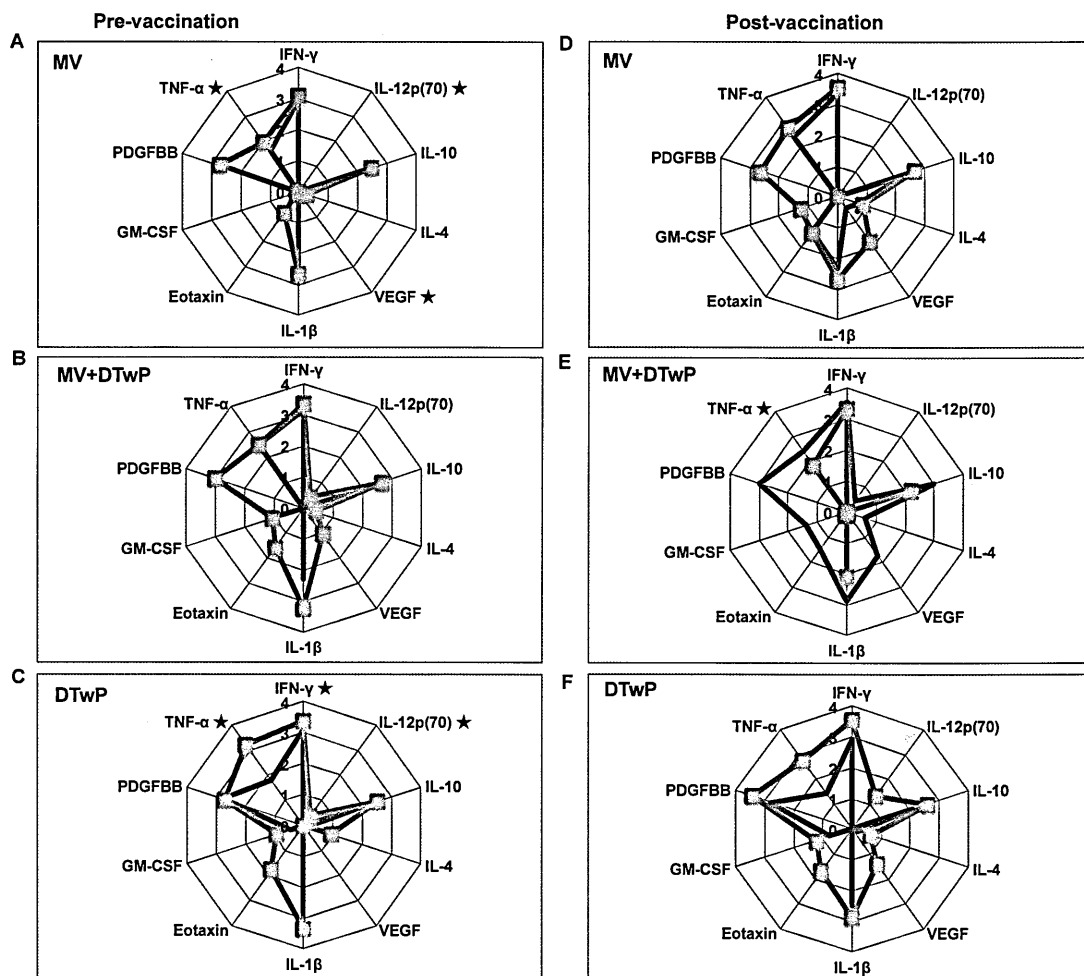


**Figure 5.11** Median cytokine levels in Measles pool cultures for IFN- $\gamma$  in the 3 vaccine groups before and after vaccination in males and females. The error bar indicates the upper quartile. P-values were derived from the GLS model analysis and indicate the vaccine time point at which there was a significant effect of sex.

### 5.3.7 Differences in PPD responses by sex

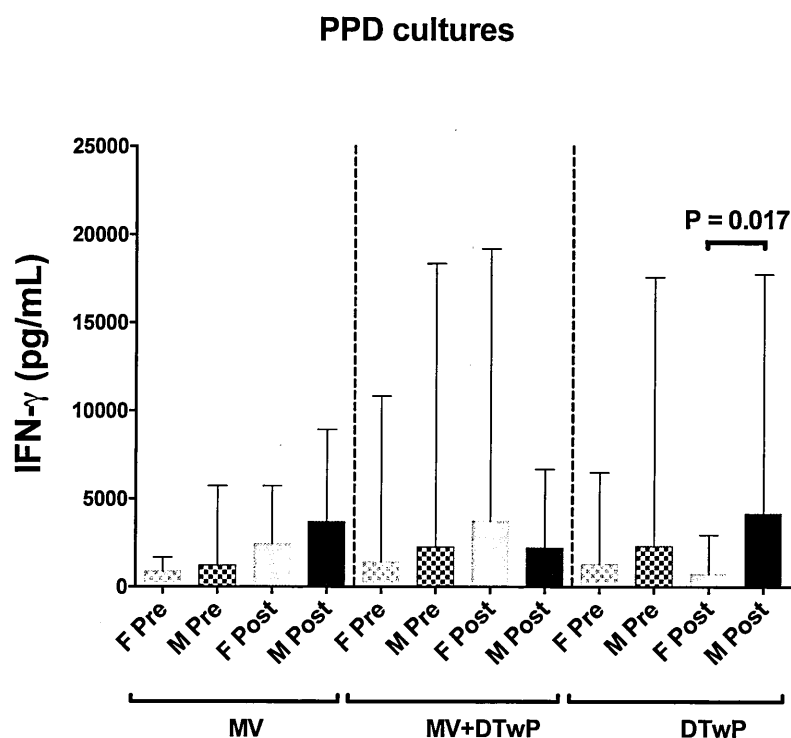
Females had lower baseline PPD reactivity than males to a number of cytokines (Table 5.5). TNF- $\alpha$  levels were lower in females compared to males in the MV and DTwP groups ( $P=0.004$ ; and  $P=0.008$  respectively; Table 5.5), while VEGF levels were lower in females compared to males in the MV group ( $P=0.018$ ; Table 5.5) (Figure 5.12A, Table 5.5); and IL-12p70, IFN- $\gamma$  and eotaxin were lower in females compared to males in the DTwP group ( $P=0.006$ ; 0.013; and 0.015 respectively; Figure 5.12C, Table 5.5).

# PPD cultures



**Figure 5.12 Spider plots showing changes in cytokine responses in PPD cultures.** Log<sub>10</sub> of all median values were plotted in the spider plots around a vertical axis creating a global view of the changes in cytokine profile in the three vaccine group before and after vaccination by sex. Purple line = responses in females and blue line = responses in males pre (A-C) and post-vaccination (D-F). All median values <1 were set to 1 to give log<sub>10</sub> value of 0. A star next to a cytokine name indicates a significant difference between males and females.

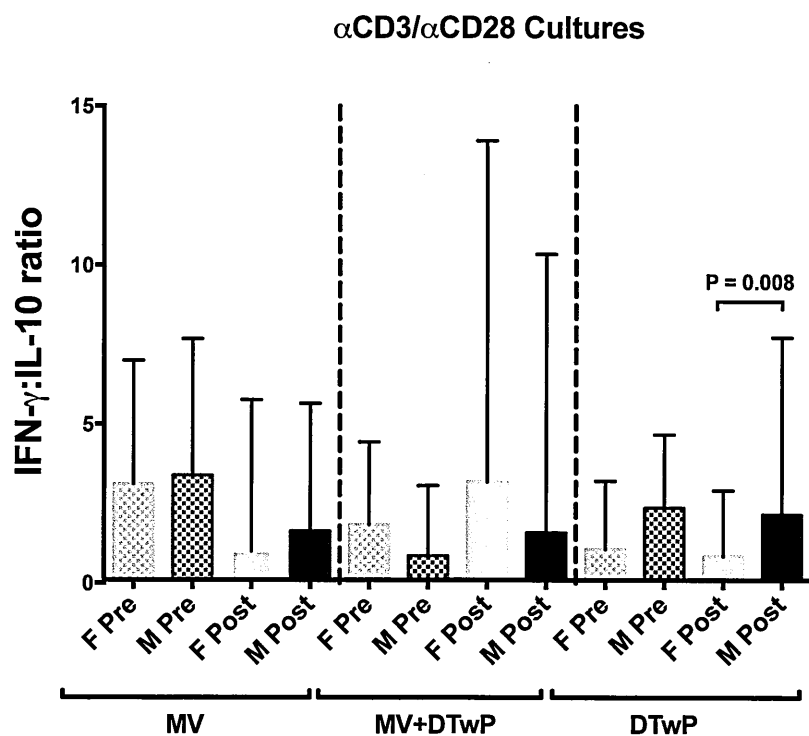
In the DTwP group the males had higher post-vaccination IFN-γ production following PPD stimulation compared to females (P=0.017; Figures 5.12F and 5.13, Table 5.5). Conversely, MV+DTwP group females had higher post-vaccination TNF-α production following PPD stimulation than males (P=0.024; Figure 5.12E, Table 5.5).



**Figure 5.13** Median cytokine levels in PPD cultures for IFN- $\gamma$  in the 3 vaccine groups before and after vaccination in males and females. The error bar indicates the upper quartile. P-values were derived from the GLS model analysis and indicate the vaccine time point at which there was a significant effect of sex.

### 5.3.8 Post-vaccination Sex Differences in the Ratio of IFN- $\gamma$ to IL-10

The ratio of the pro- to anti-inflammatory cytokines may be a better predictor of the outcome of an immune challenge than simply analysing individual cytokine levels. We therefore analyzed the ratio of IFN- $\gamma$  to IL-10 using the raw data without background subtraction to avoid negative ratios, which are not informative. The data was analyzed using GLS regression modelling. The only difference found was that DTwP vaccinated females had significantly lower IFN- $\gamma$  to IL-10 ratios post-vaccination following  $\alpha$ CD3/ $\alpha$ CD28 stimulation than DTwP males (P=0.008; Figure 5.14).



**Figure 5.14 IFN- $\gamma$  to IL-10 ratios in  $\alpha$ CD3/ $\alpha$ CD28 stimulated cultures.** Median cytokine levels in  $\alpha$ CD3/ $\alpha$ CD28 cultures for IFN- $\gamma$ : IL-10 ratios in the 3 vaccine groups before and after vaccination in males and females. The error bar indicates the upper quartile. P-values were derived from the GLS model analysis.



**Table 5.5 Summary of sex differences in the different stimulations**

| Stimulation   | Group   | Cytokine      | P-value | Responses |
|---|---------|---------------|---------|-----------|
| Interactions between group and time of vaccination in males and females - Pre-vacc  |         |               |         |           |
| PPD   | DTwP    | Eotaxin       | 0.015   | M>F       |
| Comparison of females versus males within each vaccine group - Pre-vacc             |         |               |         |           |
| PPD   | MV      | IL-12p(70)    | 0.006   | F>M       |
|   |         | TNF- $\alpha$ | 0.004   | M>F       |
|   |         | VEGF          | 0.018   | M>F       |
|   | DTwP    | IL-12p(70)    | 0.006   | M>F       |
|   |         | IFN- $\gamma$ | 0.013   | M>F       |
|   |         | TNF- $\alpha$ | 0.013   | M>F       |
| Interactions between group and time of vaccination in males and females - Post-vacc |         |               |         |           |
| Measles   | MV      | IFN- $\gamma$ | 0.031   | M>F       |
| PPD   | DTwP    | IFN- $\gamma$ | 0.017   | M>F       |
| $\alpha$ CD3/ $\alpha$ CD28   | DTwP    | IL-1 $\beta$  | 0.028   | M>F       |
| Comparison of females versus males within each vaccine group - Post-vacc            |         |               |         |           |
| PPD   | MV+DTwP | TNF- $\alpha$ | 0.024   | F>M       |
| TT  | MV      | IL-10         | 0.027   | M>F       |
|   |         | GMCSF         | 0.002   | M>F       |
|   |         | PDGFBB        | 0.003   | M>F       |
|   |         | TNF- $\alpha$ | 0.030   | M>F       |

*Summary of the interactions between group and time of vaccination by sex, as well as direct comparison between males and females within each vaccine group, for the different stimulations from the GLS regression modeling results. Stimulations in which we found a significant effect of sex in a groups' response at the pre or post-vaccination time point are indicated. P-values <0.035 were considered significant to account for FDR and are indicated.*

## 5.4 Discussion

In this chapter, we investigated the effects of giving DTwP with measles vaccine (MV+DTwP), MV alone or DTwP alone to 9-month old infants on cytokine production to vaccine-specific and non-vaccine antigens 4 weeks later. Overall a number of vaccine group and sex differences were observed following administration of the different vaccines, but these need to be interpreted with caution given the wide variation in responses between individuals, and the existence of group and sex differences prior to vaccination too.

#### 5.4.1 Vaccine-specific effects

MV is thought to induce an early pro-inflammatory IFN- $\gamma$  response in humans (Pauksen et al., 1997) followed by a later Th2 profile (Griffin and Ward, 1993). In concordance with this, those infants who received MV at 9 months (MV group and MV+DTwP group) had significantly increased IFN- $\gamma$  production in measles peptide cultures than the DTwP group who did not receive MV; while IL-4 reactivity to measles peptides was negligible, suggesting a type 1 bias to the early measles specific response following infant vaccination at 9 months.

Infants given DTwP at the same time as MV had increased post-vaccination levels of IL-10, TNF- $\alpha$ , GM-CSF and PDGF-BB in response to TT stimulation *in vitro* compared to the MV group, whereas responses were comparable to the DTP alone group. This indicates a boosting of TT-specific innate reactivity in the 2 groups that received a DTwP booster compared to the group that did not.

The MV males had greater post-vaccination TNF- $\alpha$ , IL-10, GMCSF and PDGF-BB production following TT stimulation than females from the same group, supporting an enhancement of these responses to an unrelated antigen in MV males but not females post vaccination. This would be in keeping with the enhanced pro-inflammatory effects following vaccination with another live vaccine, BCG, both in adults (Kleinnijenhuis et al., 2012) and neonates (Jensen et al., 2014). In the latter study the females were more susceptible to the enhancing effects of BCG than males, while we found the opposite for MV. In the group given MV with DTP males and females had similar responses, although the groups cannot be compared for TT reactivity since this group had a DTwP boost at 9 months, whereas the MV group did not.

#### 5.4.2 Heterologous effects on $\alpha$ CD3/ $\alpha$ CD28 and PPD responses

Responses were examined to stimulation with soluble  $\alpha$ CD3/ $\alpha$ CD28, which stimulates and expands CD4 and CD8 T cells *in vitro*, allowing them to release cytokines in an

MHC unrestricted manner. These responses were similar for all vaccine groups when males and females were analysed together. However, when separated by sex we found that the DTwP vaccinated females had down regulated IL-1 $\beta$  cytokine production and lower IFN- $\gamma$ :IL-10 ratios following  $\alpha$ CD3/ $\alpha$ CD28 stimulation compared to DTwP vaccinated males, suggesting an immunosuppressive effect of DTwP vaccination in females. DTwP vaccination has been implicated in many studies as having possible deleterious effects in females, with increased susceptibility to non-vaccine related infections and increased all-cause mortality (Aaby et al., 2004a; Aaby et al., August 2006; Benn and Aaby, 2012). A T cell immunosuppressive effect of DTwP could potentially enhance disease susceptibility, and this warrants further investigation.

Similar to their vaccine-specific responses, both the MV and the DTwP vaccinated males had a more pro-inflammatory profile with greater IL-1 $\beta$  +/- IFN- $\gamma$  following stimulation with  $\alpha$ CD3/ $\alpha$ CD28 compared to females, but this was not the case in the MV+DTwP group. This suggests a modification of this effect when the 2 vaccines are administered together, which supports the epidemiological data which shows that NSE of the individual vaccines are lost or modified when vaccines are given together.

The MV+DTwP had a more pro-inflammatory profile (IL-12p70 and TNF- $\alpha$ ) to PPD at the prevaccination baseline than the MV group. All groups were vaccinated with BCG in the first week of life; but the MV group had received 3 doses of DTwP compared to only 2 in the MV+DTwP group, so this may have played a modulatory role. Alternatively this may be due to differences in exposure to environmental mycobacteria and /or tuberculosis leading to baseline differences. Following vaccination, the reverse was observed in that the MV alone group had higher PPD-stimulated TNF- $\alpha$  reactivity than the MV+DTwP group, suggesting a vaccine modifying effect. When males and females were analysed separately, PPD stimulated TNF- $\alpha$  responses were higher in females than males in the MV+DTwP group, supporting sex-specific modification of heterologous responses when MV is given with DTwP.

At pre-the vaccination baseline, males had more IFN- $\gamma$ , TNF- $\alpha$ , IL-12p(70) and VEGF production following PPD stimulation in the MV and DTwP groups, and this baseline sex difference was retained post-vaccination in the DTP group with males having higher IFN- $\gamma$  to PPD than females.

In females, the effects observed when DTwP was given alone did not occur when DTwP was given with MV, supporting our hypothesis that combining MV with DTwP alters the immune profile. Males had more pro-inflammatory responses than females to non-specific antigens both before vaccination and after MV or DTwP, but not after combined MV+DTwP vaccination when females produced more TNF- $\alpha$  to PPD than males. TNF- $\alpha$  is a key innate pro-inflammatory cytokine to infectious challenge. It is mainly produced by activated macrophages, although CD4<sup>+</sup> T cells and NK cells also produce it. A previous Gambian study showed a relative risk of death of 5.59 (2.10; 14.8) for children having a third DTwP (DTP3) together with MV compared with children having MV only (Aaby et al., 2006b). A study from Congo showed a mortality rate ratio (MRR) of 5.38 (1.37; 21.15) (Aaby et al., 2006a) and from Malawi of 5.27 (1.11; 25.0) for children who received DTP together with MV compared with children who received only MV (Aaby et al., 2006). Alterations in pro- or anti-inflammatory responses to non-vaccine antigens could provide a possible explanation as to why vaccines might exert altered susceptibility to unrelated infections. Further longitudinal analyses of the pro- and anti-inflammatory cytokine profiles / ratios would be needed to fully understand the dynamics of the response to vaccination in the different groups and sexes.

#### **5.4.3 Study Limitations**

There are a number of limitations in this study. We were only able to assess supernatant cytokine levels after overnight culture rather than longer term, and thus most of the responses we describe in this chapter are innate or very early T cell responses. Longer culture periods would have provided more robust T cell reactivity. Furthermore these are

whole blood cultures and we don't know which cells are producing the cytokines putatively affected by vaccination. Another limitation is that we did not include more unrelated antigens due to the small sample volume available. Furthermore, the relatively small sample size, large number of cytokines and culture conditions tested, and huge inter-donor variability characteristic of human studies of this nature make it difficult to detect convincing statistically significant changes between groups. There were a number of group and sex differences at baseline sampling, maybe in part due to differences in the DTwP dosing schedule at 4 months, but also due to normal human variation due to differences in exposure to other environmental antigens by 9 months of age, and due to genetic differences.

#### **5.4.4 Overall conclusions**

Our data indicate that the different vaccine schedules result in different cytokine profiles following stimulation with vaccine-specific and non-specific antigens.

The vaccine-specific responses were as expected; boosted measles-specific IFN- $\gamma$  reactivity in those that had MV; and boosted pro-inflammatory cytokine and IL-10 TT reactivity in those that had DTwP.

The DTwP females have suppressed pro-inflammatory T cell reactivity, and lower pro-inflammatory responses to PPD than males. MV male infants have enhanced reactivity to unrelated antigens, but not when MV was given with DTwP, suggesting a dampening effect of DTwP on the pro-inflammatory effects of MV. These data are subject to the limitations described above, but lend support to sex-differential effects of vaccines on immunity to unrelated antigens. They also provide potential explanations for the epidemiological observations of deleterious effects of DTwP in females; the beneficial effects of MV; and the fact that these effects are modified when both vaccines are administered together. The results pave the way for future hypothesis driven analyses of

the mechanisms of the heterologous effects of these vaccines both in animal models and human studies.

## **CHAPTER 6**

### **POLYCHROMATIC FLOW CYTOMETRIC EVALUATION OF INNATE AND ADAPTIVE IMMUNE RESPONSES TO VACCINE SPECIFIC AND NON-VACCINE ANTIGENS IN INFANTS: INFLUENCE OF VACCINE SCHEDULE AND SEX**

## **6.1 Introduction**

The previous chapter describes the cytokine production in whole blood cultures following stimulation with vaccine-specific and non-vaccine antigens. Whilst this provides the pattern of cytokines released, it provides no information regarding the cellular source of the cytokines. The development and refinement of polychromatic flow cytometry and intracellular cytokine staining (ICS) technology allows for the phenotyping of cells producing one or more cytokines. Since we have hypothesized that the divergent heterologous effects of measles vaccine (MV) and the combined diphtheria, tetanus, whole cell pertussis vaccine (DTwP) could be related to differential immune skewing, we set out to further investigate the cellular source of type 1 (IFN- $\gamma$ , IL-2) and type 2 (IL10, IL-13) cytokines. The present chapter focuses on the production of these cytokines by CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and CD56<sup>+</sup> innate cells. Since the heterologous effect of MV and DTwP are more pronounced in females, we further analysed by sex to assess for differences in cell phenotype and function in males and females.

## **6.2 Methods**

### **6.2.1 Blood Cultures and Cell Staining**

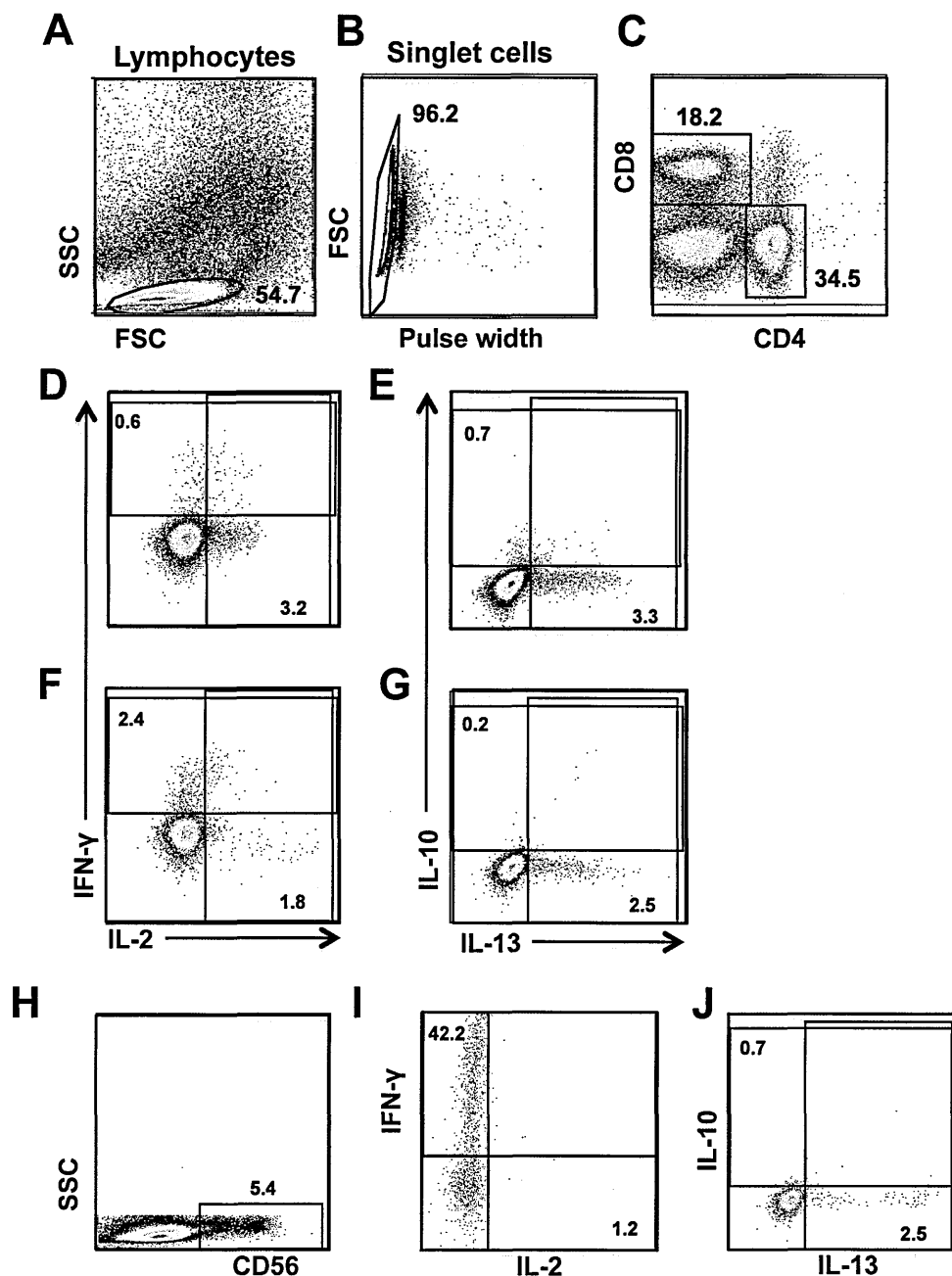
Whole blood samples from study children at 9 and 10 months of age were stimulated overnight with vaccine specific antigens (measles haemagglutinin antigen (HA) peptide pool and tetanus toxoid (TT)); the non-vaccine specific recall antigen purified protein derivative (PPD); and the TLR4 agonist lipopolysaccharide (LPS). The measles HA antigen peptide pool was used to analyse for vaccine-specific reactivity to measles, and TT was used to analyse for vaccine-specific responses to the tetanus toxoid component of DTwP vaccine. Anti-CD3/CD28 was used as a positive control and medium alone served as the negative control for spontaneous cytokine production (background). Intracellular cytokine staining (ICS) was performed as described in Chapter 2. Briefly, whole blood in 96-well plates was cultured at 37°C, 5% CO<sub>2</sub> for 16 hours with the addition of Brefeldin A after the first 2 hours of culture. Cells were then surface stained with the antibodies anti-CD4 APC-Cy7 and anti-CD8 PerCP for detection of CD4<sup>+</sup> and CD8<sup>+</sup> T cells respectively and anti-



CD56 PE-Cy7 for detection of innate cells. After fixation and permeabilization, cells were stained intracellularly with antibodies to IFN- $\gamma$  APC, IL-2 PE, IL-10 PB and IL-13 FITC.

### **6.2.2 ICS Gating Strategy**

Cells were acquired using a CyAn ADP™ 9-colour flow cytometer (Beckman Coulter, USA). Lymphocytes were gated according to FSC and SSC and compensation performed. At least 100,000 lymphocytes were acquired and analysis was performed using FlowJo software version 9.4.7 (Treestar, USA). From the lymphocyte gate (Figure 6.1A), we gated on single cells using forward scatter and pulse width (Figure 6.1B). From the singlet population we then gated on CD4+, CD8+ and CD56+ cells (Figure 6.1C and 6.1H). From each of these cell populations we then gated on the cytokine-positive population using Boolean gating. This allowed us to determine combinatorial cytokine production using FlowJo. Grouped analysis of Th1 (IL-2 and IFN- $\gamma$ ) or Th2 (IL10 and IL13) was performed.



**Figure 6.1** *Gating strategy for cytokine producing cells.* Lymphocytes were gated using side and forward scatter (A). Singlets were then gated using forward scatter and pulse width (B). CD4<sup>+</sup>, CD8<sup>+</sup> (C) and CD56<sup>+</sup> cells (H) were then gated and analysed for the proportion of cytokine producing cells within each subset using Boolean gating (D-G, I-J).

After determining the percentages of CD4<sup>+</sup>, CD8<sup>+</sup> and CD56<sup>+</sup> cells that expressed intracellular IFN- $\gamma$ , IL-2, IL-10, IL-13 or a combination of these cytokines, background cytokine responses from medium (negative control) cultures were subtracted from antigen stimulated cytokine responses. All net values  $\leq 0.01\%$  were given a value of zero since this

was assumed to represent background production, as previously described (Hanekom et al., 2004).

### **6.2.3 Statistical Analysis**

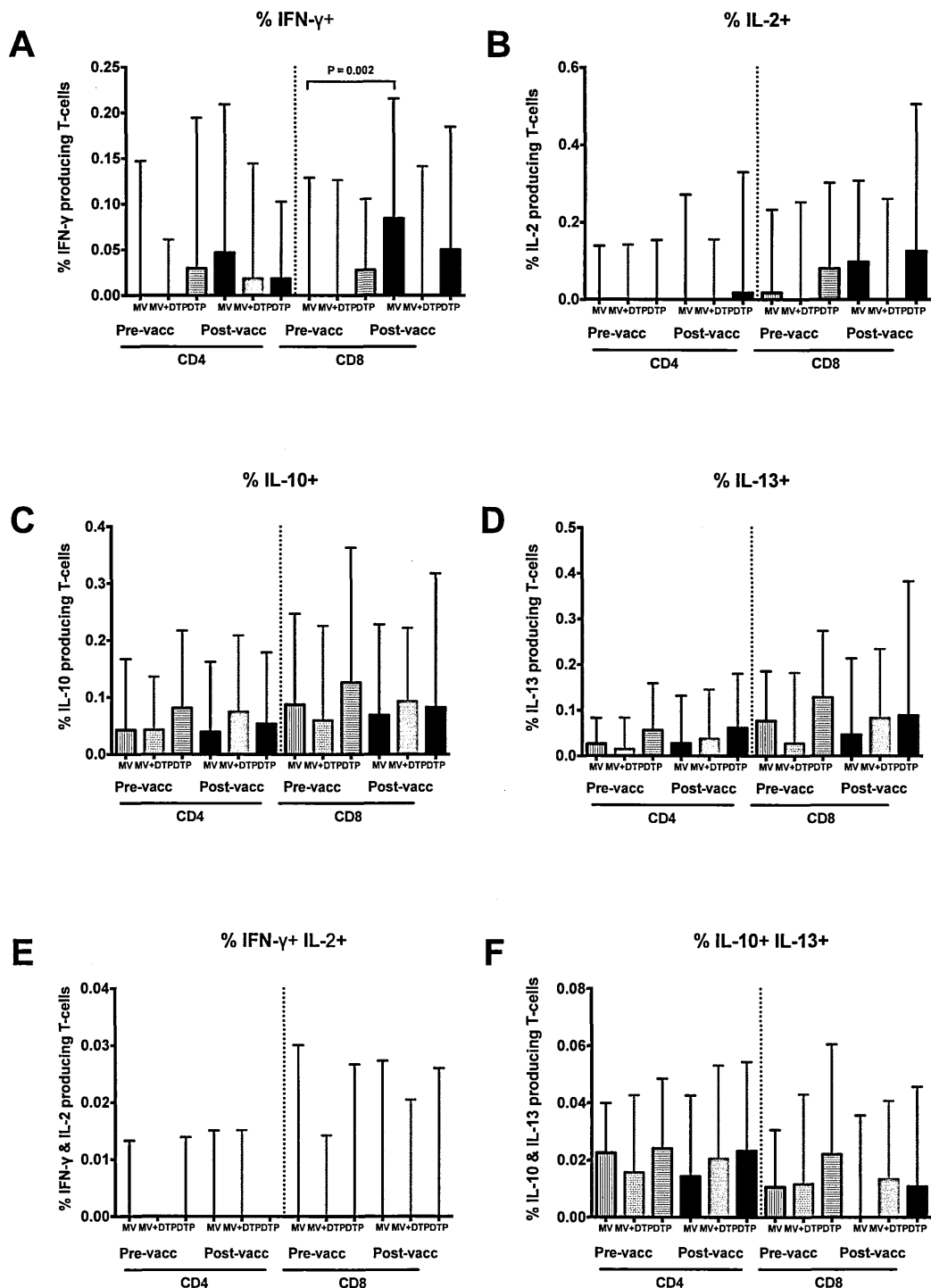
Differences between the 3 vaccine groups pre- and post-vaccination were analysed using a Kruskal-Wallis test followed by Dunn's test after which the P-values were corrected for multiple test comparison using False Discovery Rates (FDR). Mann-Whitney U-tests were used to determine differences between pre- and post-vaccination responses for each group and between males and females within each group. P-values <0.035 were considered significant to account for FDR.

## **6.3 Results**

### **6.3.1 Measles-Specific CD4+ and CD8+ T Cell Responses**

Following stimulation with measles antigen (measles peptide pool), there were no differences in the frequency of CD4+IFN- $\gamma$ + T cells between the groups pre- or post-vaccination (Figure 6.2A, Table 6.1). The proportion of CD8+IFN- $\gamma$ + T cells was significantly increased post-vaccination in the MV group compared to pre-vaccination levels (P=0.002; Figure 6.2A, Table 6.1); no differences were seen in the other vaccine groups.

Measles antigen cultures



**Figure 6.2** T cell frequencies in the MV, MV+DTwP and DTwP groups (patterned bars) pre-vaccination and post-vaccination (filled bars) in the measles stimulated cultures. (A) Shows the frequency of IFN- $\gamma$ + CD4+ and CD8+ T cells, (B) shows the frequency of IL-2+ CD4+ and CD8+ T cells, (C) shows the frequency of IL-10+ CD4 and CD8+ T cells, (D) shows the frequency of IL-13+ CD4 and CD8+ T cells, (E) shows the frequency of IFN- $\gamma$ +IL-2+ CD4+ and CD8+ T cells and (F) shows the frequency of IL-10+IL-13+ CD4+ and CD8+ T cells. The error bar indicates the upper quartile. P-values were generated using Mann-Whitney U-test for differences between pre- and post-vaccination frequencies within the same vaccine group, and ANOVA with Dunn's test for differences across all groups. P-values <0.035 were considered significant to account for FDR.

There was no difference in the percentage of measles pool stimulated IL-2+, IL-10+ or IL-13+ CD4+ or CD8+ cells following vaccination (Figure 6.2B, C and D, Table 6.1). There was no significant difference in the proportion of double positive (IFN- $\gamma$ +IL-2+ or IL-10+IL-13+) cells in any of the groups in measles pool cultures after vaccination (Figure 6.2E and F).

The proportions of CD4+ T cells producing both IL-10 and IL-13 in measles cultures was significantly higher in males (median 0.04, IQR 0-0.06) in the DTwP group compared to females (median 0.01, IQR 0-0.04) post-vaccination ( $P=0.028$ ; Table 6.2). All infants in the three vaccine groups were measles naïve pre-vaccination and the DTwP group remained naïve as they only received DTwP.

### **6.3.2 TT-Specific CD4+ and CD8+ T cell Responses**

The only difference in TT responses was a significantly increased proportion of IL-13+CD8+ T cells following vaccination in the MV+DTwP group (median 0.05, IQR 0-0.27) ( $P=0.017$ ; Table 6.1). This was not observed in the DTwP alone group.

We further investigated whether sex has an effect on T cell responses to TT stimulation. MV+DTwP group females had a significantly higher proportion of CD4+IFN- $\gamma$ +IL-2+ T cells compared to MV+DTwP males pre-vaccination ( $P=0.003$ ; Table 6.2); but no difference was seen post-vaccination. Following vaccination, DTwP males had a higher proportion of CD4+IL-10+IL-13+ T cells compared to females ( $P=0.011$ ; Table 6.2).

### **6.3.3 CD4+ and CD8+ T Cell Responses to the Broad T Cell Stimulant Anti-CD3/anti-CD28**

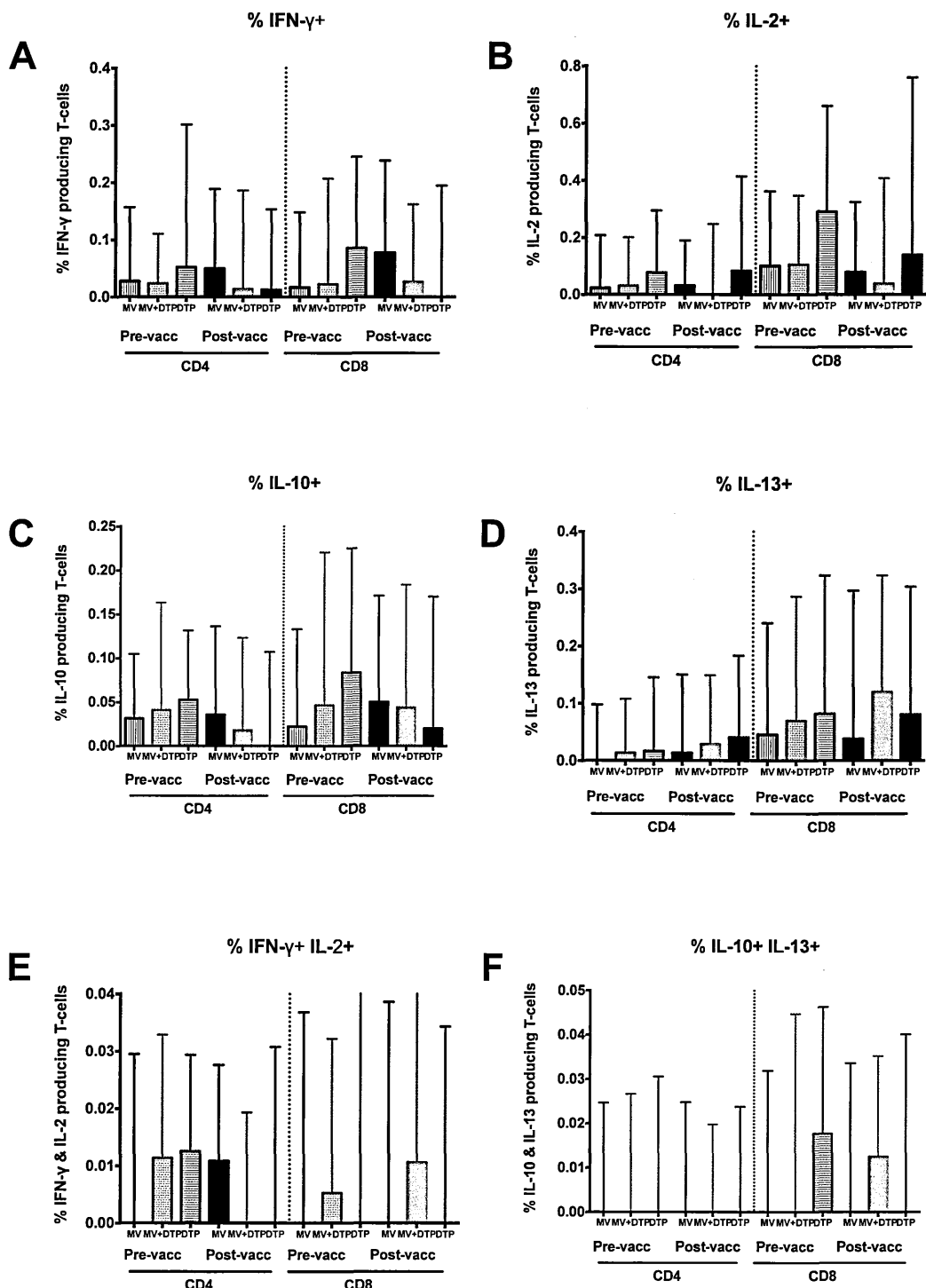
Any effects of vaccination on responses to the general T cell stimulant anti-CD3/CD28 (positive control) would suggest a broad immune modulatory effect. The DTwP vaccinated infants had a decreased proportion of CD4+IFN- $\gamma$ + ( $P=0.002$ ), CD4+IFN- $\gamma$ +IL-2+ double positive ( $P=0.021$ ) and CD4+IL-10+ ( $P=0.008$ ) cells post-vaccination compared to pre-

vaccination levels (Table 6.1). There was also a decrease in the proportion of CD8+IL-2+ cells post-vaccination in DTwP vaccinated individuals compared to pre-vaccination levels (median 0.42, IQR 0.01-0.95 and median 0.11, IQR 0.0-0.05, respectively,  $P=0.027$ ). No sex differences were observed for anti-CD3/CD28 responses within or between groups (Table 6.2).

#### **6.3.4 CD4+ and CD8+ T cell Responses to PPD**

BCG was administered to all children in the first week of life as part of the Gambian EPI vaccine schedule and thus they might be expected to have *in vitro* PPD reactivity at 9 months of age which could also be due to exposure to environmental mycobacteria. We found no difference in responses to PPD by ICS for CD4+ or CD8+ T cells for single cytokine positive cells (IFN- $\gamma$ , IL-2, IL-10 or IL-13) or IL-10+IL-13+ double cytokine positive cells (Figure 6.3 A-D and H) either between vaccine groups or pre to post comparison within the same group. As expected, there was higher production of IFN- $\gamma$  from CD4+ T cells than from the CD8+ T cells (Figure 6.3A).

# PPD antigen cultures



**Figure 6.3** T cell frequencies in the MV, MV+DTwP and DTwP group (patterned bars) pre-vaccination and post-vaccination (filled bars) in the PPD stimulated cultures. (A) The frequency of IFN-γ+ CD4+ and CD8+ T cells; (B) The frequency of IL-2+ CD4+ and CD8+ T cells; (C) The frequency of IL-10+ CD4 and CD8+ T cells; (D) The frequency of IL-13+ CD4 and CD8+ T cells; (E) The frequency of IFN-γ+IL-2+ CD4+ and CD8+ T cells; (F) The frequency of IL-10+IL-13+ CD4+ and CD8+ T cells. The error bar indicates the upper quartile. P-values were generated using Mann-Whitney U-test for differences between pre- and post-vaccination frequencies within the same vaccine group, and ANOVA with Dunn's test for differences across all groups. P-values <0.035 were considered significant to account for FDR.

There were no differences in CD4+ T cell responses between males and females following PPD stimulation. However, the proportion of CD8+IL-13+ cells was significantly higher in MV males compared to MV females post-vaccination (P=0.008; Table 6.2)

**Table 6.1 Summary of the significant differences in T cell frequencies between vaccine groups and between pre- and post-vaccination time points for the different stimulations**

| Antigen (Stimulant)                          | Vaccine Group | Cytokine+ cells | Pre-vacc<br>(IQ Range) | Post-vacc<br>(IQ Range) | P-value | Responses<br>↑ or ↓ |
|--|---------------|-----------------|------------------------|-------------------------|---------|---------------------|
| Pre vs Post-vaccination                      |               |                 |                        |                         |         |                     |
| CD4  |               |                 |                        |                         |         |                     |
| αCD3/αCD28                                   | DTwP          | IFN-γ+          | 0.09<br>(0 – 0.47)     | 0<br>(0 – 0.11)         | 0.002   | Pre > Post          |
|  |               | IL-10+          | 0.12<br>(0 – 0.26)     | 0.04<br>(0 – 0.14)      | 0.008   | Pre > Post          |
|  |               | IFN-γ+IL-2+     | 0<br>(0 – 0.04)        | 0<br>(0 – 0.01)         | 0.021   | Pre > Post          |
| CD8  |               |                 |                        |                         |         |                     |
| Measles                                      | MV            | IFN-γ+          | 0<br>(0 – 0.13)        | 0.08<br>(0 – 0.22)      | 0.002   | Post > Pre          |
| TT   | MV+DTwP       | IL-13+          | 0.01<br>(0 – 0.08)     | 0.05<br>(0 – 0.27)      | 0.017   | Post > Pre          |
| αCD3/CD28                                    | DTwP          | IL-2+           | 0.42<br>(0.01 – 0.95)  | 0.15<br>(0 – 0.55)      | 0.027   | Pre > Post          |
| Between Group Differences (Post-vaccination) |               |                 |                        |                         |         |                     |
| CD4  |               |                 |                        |                         |         |                     |
| αCD3/αCD28                                   | MV vs DTwP    | IFN-γ+          | 0.09<br>(0 – 0.39)     | 0<br>(0 – 0.11)         | 0.002   | MV > DTwP           |

The table shows median values from stimulations in which we found significant differences. ANOVA with Dunn’s multiple comparison tests was used for differences across all groups. P-values are reported on the table and were generated using Mann-Whitney U-test for differences between pre- and post-vaccination frequencies within the same vaccine group. P-values <0.035 were considered significant to account for FDR and are indicated.



**Table 6.2 Summary of significant differences in T cell and NK cell frequencies between males and females pre- and post-vaccination for the different stimulations**

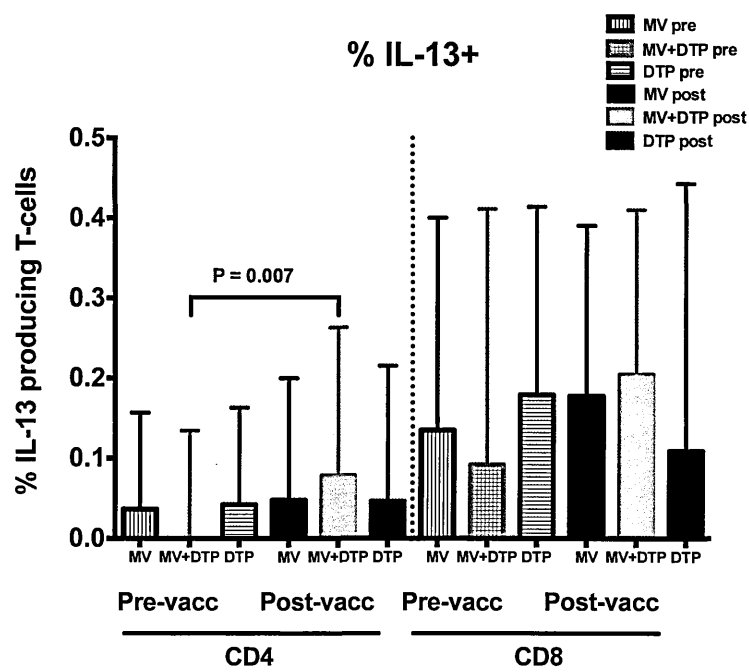
| Pre-vaccination sex differences  |               |                      |                      |                    |         |                |
|----------------------------------|---------------|----------------------|----------------------|--------------------|---------|----------------|
|                                  | Vaccine group | Cytokine+ cells      | Pre-vacc (IQ range)  |                    | P-value | Sex difference |
|                                  |               |                      | M                    | F                  |         |                |
| CD4                              |               |                      |                      |                    |         |                |
| TT                               | MV+DTwP       | IFN- $\gamma$ +IL-2+ | 0<br>(0 – 0.00)      | 0<br>(0 – 0.03)    | 0.003   | F>M            |
| PPD                              | MV+DTwP       | IL-2+                | 0<br>(0 – 0.14)      | 0.11<br>(0 – 0.27) | 0.021   | F>M            |
| LPS                              | MV+DTwP       | IL-2+                | 0<br>(0 – 0.09)      | 0.06<br>(0 – 0.39) | 0.005   | F>M            |
| CD56                             |               |                      |                      |                    |         |                |
| TT                               | MV+DTwP       | IL-2+                | 0<br>(0 – 0.33)      | 0.32<br>(0 – 0.93) | 0.013   | F>M            |
| TT                               | DTwP          | IL-10+               | 0.09<br>(0 – 0.46)   | 0<br>(0 – 0.05)    | 0.018   | M>F            |
| PPD                              | MV+DTwP       | IL-13+               | 0<br>(0 – 0.35)      | 0.04<br>(0 – 1.32) | 0.023   | F>M            |
| Post-vaccination sex differences |               |                      |                      |                    |         |                |
|                                  | Vaccine group | Cytokine+ cells      | Post-vacc (IQ range) |                    | P-value | Sex difference |
| CD4                              |               |                      |                      |                    |         |                |
| Measles                          | DTwP          | IL-10+IL-13+         | 0.04<br>(0 – 0.06)   | 0.01<br>(0 – 0.04) | 0.028   | M>F            |
| TT                               | DTwP          | IL-10+IL-13+         | 0.01<br>(0 – 0.03)   | 0<br>(0 – 0.01)    | 0.011   | M>F            |
| LPS                              | DTwP          | IL-10+IL-13+         | 0.02<br>(0 – 0.07)   | 0<br>(0 – 0.02)    | 0.009   | M>F            |
| CD8                              |               |                      |                      |                    |         |                |
| PPD                              | MV            | IL-13+               | 0.06<br>(0 – 0.40)   | 0<br>(0 – 0.09)    | 0.008   | M>F            |
| CD56                             |               |                      |                      |                    |         |                |
| aCD3/CD28                        | DTwP          | IFN- $\gamma$ +      | 1.49<br>(0 – 3.51)   | 0<br>(0 – 0.66)    | 0.014   | M>F            |
| PPD                              | DTwP          | IL-10+               | 0<br>(0 – 0.35)      | 0.31<br>(0 – 0.91) | 0.025   | F>M            |

Significant differences in CD4+ and CD8+ T cell and CD56+ cell frequencies between males and females in MV group, MV+DTwP group and DTwP group pre-vaccination and post-vaccination. The table shows median values from stimulations in which we found significant sex differences. P-values were generated using Mann-Whitney U-test for differences between males and females pre-and post-vaccination frequencies within the same vaccine group, followed by Dunn's multiple comparison test for differences across all groups. P-values <0.035 were considered significant to account for FDR and are indicated.

### 6.3.5 T Cell Responses to LPS (TLR4 Agonist) Stimulation

TLR agonists have been shown to increase adaptive immune responses in infants (Wu et al., 2004; Wille-Reece et al., 2005) and have the potential to boost vaccine efficacy.

Therefore we wanted to determine if the different vaccine groups responded differently to TLR agonist stimulation. Following LPS (TLR4 agonist) stimulation, there were no significant differences between the groups for single-positive for IFN- $\gamma$ , IL-2 and IL-10 or dual-positive (IFN- $\gamma$ +IL-2+ or IL-10+IL-13+) CD4+ or CD8+ T cells before or after vaccination when all donors were analysed together. Overall there was a greater production of IL-13 by CD8+ T cells than the CD4+ population in LPS cultures both pre- and post-vaccination (Figure 6.4). The frequency of CD4+IL-13+ cells was significantly higher post-vaccination compared to pre-vaccination in the MV+DTwP group ( $P=0.007$ ; Figure 6.4, Table 6.4).



**Figure 6.4** Frequencies of IL-13+ CD4 and CD8+ T cells in the MV, MV+DTwP and DTwP group (patterned bars) pre-vaccination and post-vaccination (filled bars) in the LPS cultures. *P*-values were generated using Mann-Whitney U-test for differences between pre- and post-vaccination frequencies within the same vaccine group and ANOVA with Dunn's post-test comparison for differences across all groups. The error bar indicates the upper quartile. *P*-values <0.035 were considered significant to account for FDR and are indicated.

In the sex analysis, there was a significantly lower proportion of CD4+IL-2+ T cells in MV+DTwP males than females pre-vaccination ( $P=0.005$ ; Table 6.2), but this was not sustained post-vaccination. The post-vaccination frequency of CD8+IFN- $\gamma$ + T cells following LPS stimulation was significantly higher in males in the MV group compared to males in both other groups ( $P=0.003$  and  $P=0.002$  respectively, Table 6.3). DTwP males

had a significantly higher proportion of CD4+IL-10+IL-13+ T cells post-vaccination compared to females (P=0.009; Table 6.2), but no sex differences were observed in the DTwP vaccinated group CD8+ T cell response (Table 6.2).

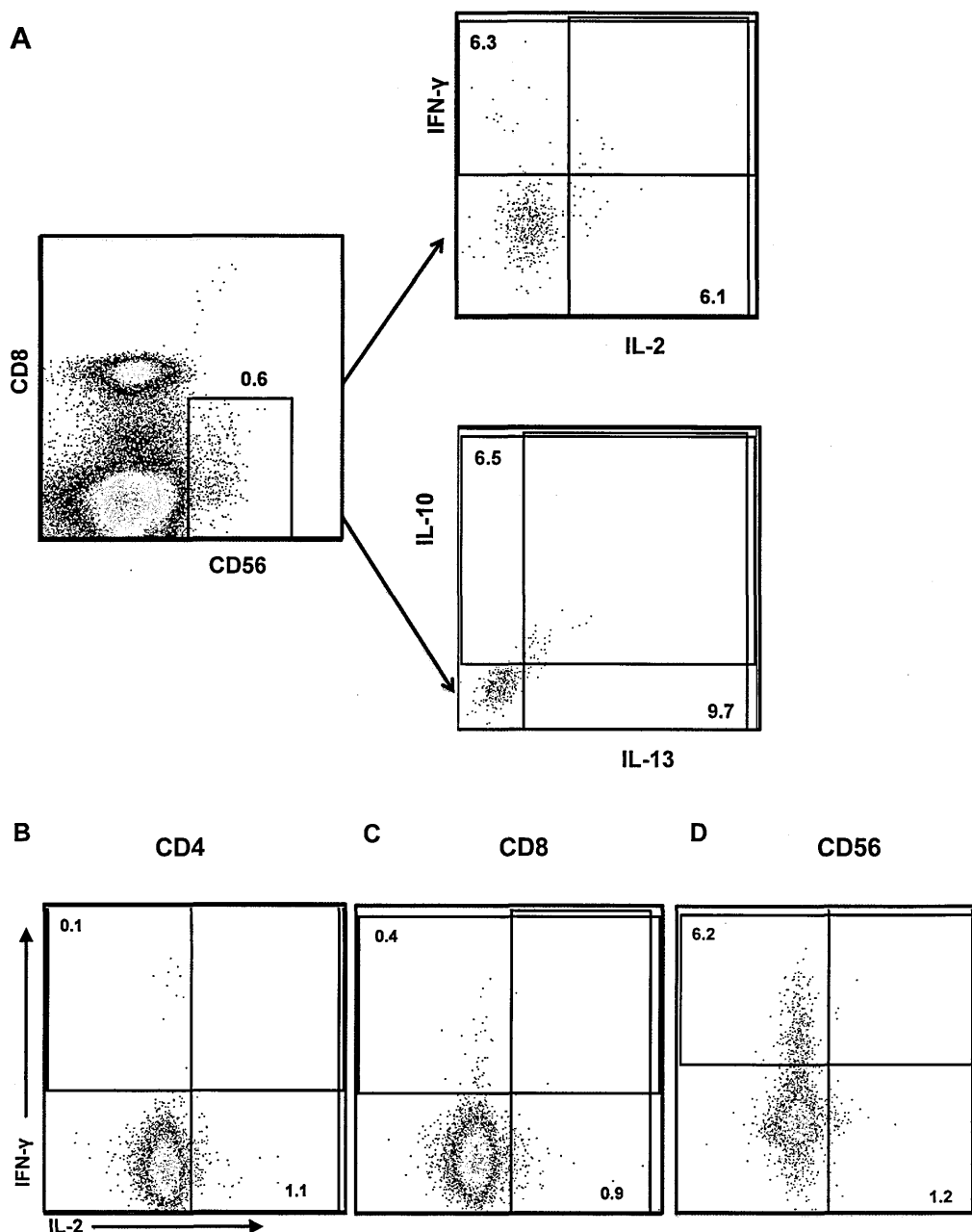
**Table 6.3 Post-vaccination group differences in T cell frequencies in LPS stimulated cultures by sex**

| Antigen<br>(Stimulant)                    | Vaccine Group | Sex   | Cytokine+ | Post-vacc<br>(IQ Range 1 <sup>st</sup> Group) | Post-vacc<br>(IQ Range 2 <sup>nd</sup> Group) | P-value | Responses<br>↑ or ↓ |
|---|---------------|-------|-----------|---|---|---------|---------------------|
| Post-vaccination Group differences by sex |               |       |           |   |   |         |                     |
| CD8                                       |               |       |           |   |   |         |                     |
| LPS                                       | MV vs MV+DTwP | Males | IFN-γ+    | 0.20<br>(0.01 – 0.40)                         | 0<br>(0 – 0.14)                               | 0.003   | MV > MV+DTwP        |
| LPS                                       | MV vs DTwP    | Males | IFN-γ+    | 0.20<br>(0.01 – 0.40)                         | 0<br>(0 – 0.15)                               | 0.002   | MV > DTwP           |

*Significant difference in CD8+ T cell frequencies in LPS cultures between MV group, MV+DTwP group and DTwP group post-vaccination when analysed by sex; median values are shown. Only comparisons with significant differences are shown for LPS cultures. P-values were generated using Mann-Whitney U-test for differences between groups in males and females. ANOVA with Dunn's multiple comparison tests was used for differences across all groups. P-values <0.035 were considered significant to account for FDR and are indicated.*

**6.3.6 High levels of cytokine production by NK cells in response to MV peptide stimulation**

IFN-γ production was more than 5-fold higher from CD56+ NK cells compared to CD4+ or CD8+T cells (Figure 6.5) following stimulation with measles peptides confirming that the bulk of the IFN-γ production detected by luminex is innate derived. The percentage of NK cells producing IFN-γ, IL-2, IL-10 or IL-13 showed no differences between the vaccine groups pre-vaccination when all donors were analysed together. No significant differences were seen when responses were analysed by sex.



**Figure 6.5** Flow cytometry plot of CD56 gated against CD8 (A) and IFN- $\gamma$  production from T cells and CD56+ cells (B-D). CD56+ cells were generally distinct from the CD8+ subset, and produced all 4 cytokines (IFN- $\gamma$ , IL-2, IL-10 and IL-13). The CD56+ population includes NK cells, NK T cells and  $\gamma\delta$  T cells. The CD56+CD8low tend to be the NKT and/or  $\gamma\delta$  T cells. Percentages of IFN- $\gamma$  production to measles antigen stimulation in CD4+ cells (B), CD8+ cells (C), and CD56+ cells (D) in one individual in response to measles antigen stimulation.

**Table 6.4 Summary of the significant differences in T cell and CD56+ cell frequencies between vaccine groups and between pre- and post-vaccination time points for the different stimulations**

| Antigen (Stimulant)            | Vaccine Group | Cytokine+ cells | Pre-vacc<br>(IQ Range) | Post-vacc<br>(IQ Range) | P-value | Responses<br>↑ or ↓ |
|--------------------------------|---------------|-----------------|------------------------|-------------------------|---------|---------------------|
| <b>Pre vs Post-vaccination</b> |               |                 |                        |                         |         |                     |
| <b>CD56</b>                    |               |                 |                        |                         |         |                     |
| TT                             | DTwP          | IL-13+          | 0.25<br>(0 – 1.00)     | 0<br>(0 – 0.33)         | 0.006   | Pre > Post          |
|                                | MV            | IL-10+IL13+     | 0<br>(0 – 0.00)        | 0<br>(0 – 0.27)         | 0.019   | Post > Pre          |
| PPD                            | MV+DTwP       | IL-2+           | 0.34<br>(0 – 1.02)     | 0<br>(0 – 0.56)         | 0.019   | Pre > Post          |
|                                | DTwP          | IL-13+          | 0.39<br>(0 – 1.23)     | 0<br>(0 – 0.53)         | 0.026   | Pre > Post          |
|                                | MV+DTwP       | IFN-γ+IL-2+     | 0<br>(0 – 0.27)        | 0<br>(0 – 0.00)         | 0.001   | Pre > Post          |
| LPS                            | MV+DTwP       | IL-2+           | 0.44<br>(0 – 1.21)     | 0<br>(0 – 0.61)         | 0.006   | Pre > Post          |
| <b>CD4</b>                     |               |                 |                        |                         |         |                     |
| LPS                            | MV+DTwP       | IL-13+          | 0<br>(0 – 0.13)        | 0.08<br>(0 – 0.26)      | 0.007   | Post > Pre          |

*Significant differences in CD4+ T cell and CD56+ cell frequencies in the MV group, MV+DTwP group and DTwP group pre-vaccination compared to post-vaccination. Results from stimulations with significant differences are shown. P-values were generated using Mann-Whitney U-test for differences between pre- and post-vaccination frequencies within the same vaccine group. P-values <0.035 were considered significant to account for FDR and are indicated.*

### 6.3.7 NK cell responses to TT Stimulation

TT reactivity in CD56+ cells was very low, with many median values being zero both before and after vaccination. There were no differences between the groups pre- or post-vaccination but the % of CD56+IL-13+ cells was significantly lower following vaccination in the DTwP group compared to pre-vaccination levels (P=0.006; Table 6.4).

In the sex analysis females had a higher percentage of CD56+IL-2+ (P=0.013) and lower CD56+IL-10+ cells (P=0.0181) than males at pre-vaccination baseline in the MV+DTwP group and the DTwP groups respectively (Table 6.2). These differences were lost post-vaccination.

### 6.3.8 NK cell responses to αCD3/αCD28 stimulation

IFN-γ production by CD56+ cells in αCD3/28 cultures was higher than in the measles, PPD and TT stimulated cultures (Figure 6.6). There were no differences in cytokine production

between the 3 groups, and no changes following vaccination in any of the groups in  $\alpha$ CD3/ $\alpha$ CD28 cultures. When analysed by sex, the percentage of CD56+IFN- $\gamma$ + cells was significantly higher in DTwP vaccinated males (median 1.49, IQR 0.0-3.51) compared to females (median 0.0, IQR 0.0-0.66) post-vaccination ( $P=0.014$ ; Table 6.2).

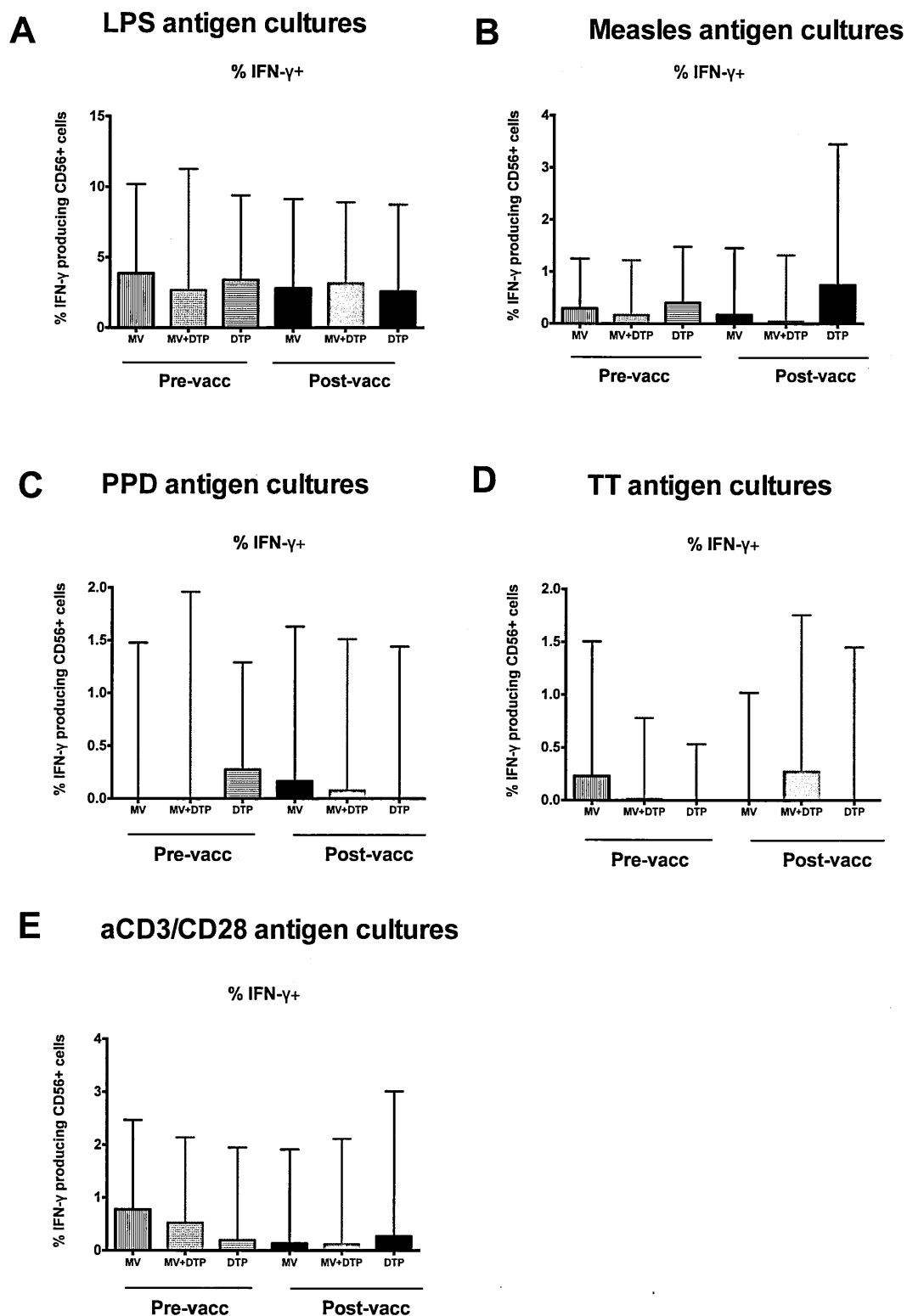
### **6.3.9 NK cell responses to PPD**

Following PPD stimulation, the proportion of CD56+IFN- $\gamma$ + cells was similar between the vaccine groups. However, the proportion of CD56+ cells producing IL-2 alone or in combination with IFN- $\gamma$  was reduced following vaccination in the MV+DTwP group ( $P=0.019$  and  $P=0.001$  respectively; Table 6.4). A decreased percentage of CD56+IL-13+ cells was also found post-vaccination in the DTwP group (median 0.0, IQR 0-0.53) compared to pre-vaccination (median 0.39, IQR 0.0-1.23;  $P=0.026$ ; Table 6.13).

The MV+DTwP females had a higher pre-vaccination proportion of CD56+IL-13+ cell frequencies in PPD cultures (median 0.04, IQR 0.0-1.32) compared to males (median 0.0, IQR 0.0-0.35) ( $P=0.023$ ; Table 6.2). In addition, the percentage of CD56+IL-10+ cells was significantly higher in DTwP vaccinated females post-vaccination (median 0.31, IQR 0.0-0.91) compared to males (median 0.0, IQR 0.0-0.35) ( $P=0.025$ ; Table 6.2).

### **6.3.10 NK cell responses to LPS (TLR4 agonist)stimulation**

IFN- $\gamma$  production by CD56+ cells was higher in LPS cultures compared to PPD, TT, measles pool and  $\alpha$ CD3/ $\alpha$ CD28 cultures (Figure 6.6). There was no difference in CD56+ cell cytokine production between groups either before or after vaccination following LPS stimulation. However, within the MV+DTwP group, proportions of CD56+IL-2+ cells following LPS stimulation decreased significantly post-vaccination (median 0.0, IQR 0.0-0.61) compared to pre-vaccination levels (median 0.44, IQR 0.0-1.21) ( $P=0.006$ ; Table 6.4). There were no sex differences in CD56+ cell frequencies in LPS cultures.



**Figure 6.6 Proportions of IFN- $\gamma$  produced by CD56+ cells.** Comparison of IFN- $\gamma$  production by CD56+ cells in the different antigen cultures (A-E) in the three vaccine groups. The error bar indicates the upper quartile.

## 6.4 Discussion

The aim of this chapter was to determine whether giving DTwP with MV, MV alone or DTwP alone altered cytokine responses following vaccine-specific, recall or TLR agonist stimulation by NK cells and T cells at the single-cell level. Production of IFN- $\gamma$ , IL-2, IL-10 and IL-13 was assessed using intracellular cytokine staining to determine the cellular source of cytokines observed in chapter 5.

### 6.4.1 Antigen Specific T Cell Effects

In measles antigen cultures, the expected enhanced Tc1 IFN- $\gamma$  CD8 T cell response was observed in the MV group 4 weeks after vaccination, but not in the DTwP vaccine recipients. This suggests that MV alone results in a predominant Tc1 CD8+ response. This was not apparent in the MV+DTwP group suggesting interference with stimulating a memory response to MV when DTwP is given simultaneously. The stronger Th2 responses (CD4+ IL-10+IL13+) in measles pool cultures in males compared to females in the DTwP group probably represents an innate response to intrinsic innate agonists in this live vaccine since this group was measles naïve.

*In vitro* TT responses were of low level, perhaps due to the relatively short culture period of 16 hours which may have been insufficient for the adequate detection of this recall antigen response. As a result there did not appear to be any boosted TT reactivity 4 weeks after vaccination in the DTwP vaccine recipients. This is in concordance with our soluble cytokine results where TT responses were low level, and the DTwP vaccinated group failed to significantly increase responses to TT compared to the MV group, although there was an enhancement for most cytokines tested in the MV+DTwP group.

### 6.4.2 Non-Specific T Cell Responses

Reactivity to the general T cell stimulus  $\alpha$ CD3/ $\alpha$ CD28 was interesting in that the DTwP group had suppressed CD4 (Th1 and Th2) T cell reactivity 4 weeks after vaccination. By contrast, the  $\alpha$ CD3/ $\alpha$ CD28 response was not affected after MV or MV+DTwP vaccination.



This was not observed in the soluble cytokine results for which there were no differences between the vaccine groups in response to the positive control stimulation when all donors were analysed together. However, in the previous chapter DTwP vaccination had a suppressive effect on supernatant IL-1 $\beta$  responses to  $\alpha$ CD3/ $\alpha$ CD28 stimulation in females, so overall the data support immunosuppressive effects of DTwP vaccination, particularly in females.

There was no effect of DTwP vaccination on PPD-specific ICS T cell responses 4 weeks later, but the MV+DTwP group had a decreased Th1 response to PPD, suggesting a decrease in a potentially protective Th1 response to tuberculosis (TB). MV males had enhanced Tc2 reactivity to PPD compared to females, which could reduce their protection against TB since type 1 cytokine (IFN- $\gamma$ ) responses are thought to be protective, although the precise correlates of TB protection are still not known. Alteration in anti-tuberculous immunity following administration of an unrelated vaccine suggests that responses to heterologous antigens might be altered by vaccination. The only other significant difference following PPD stimulation was a lower frequency of CD4+IL-2+ T cells in MV+DTwP males compared to females pre-vaccination (Table 6.2), but not post-vaccination. We observed similar findings in the previous chapter in which the luminex profiles also showed more differences in PPD reactivity pre-vaccination. Pre-vaccination sex differences in PPD reactivity in 1 group only could be due to baseline group differences in TB / mycobacterial exposure, group differences in BCG reactivity, but could also be due to chance and may not be a truly significant finding.

#### **6.4.3 Effects on Innate Immune Responses to Vaccine Antigens**

Innate immunity was assessed by gating on all CD56+ cells and looking at their intracellular cytokine profile; and by stimulating with the TLR4 agonist LPS and analyzing ICS cytokine percentages in CD56+ cells and also T cells. The marker CD56 was used to detect NK cells, although strictly speaking these should be further subdivided into CD56<sup>bright</sup> and CD56<sup>dim</sup>, and the markers CD16 and CD8 may be further used to differentiate between

various NK cell populations (Reviewed in (Poli et al., 2009)). Due to limits on the number of fluorochromes that could be tested, the marker CD3 was not available in the panel to confirm that the cells were truly CD3 negative and therefore NK cells. Thus by simply gating on all CD56+ cells there was likely a mix of NK and NK T cells represented in the subset analysed in this study. CD3<sup>bright</sup>CD56+ cells are typical of  $\gamma\delta$ T cells, which are likely to be more abundant in infants than adults and thus these would also be represented in our gating strategy. Thus by gating on CD56+ cells we are potentially describing cytokine production from NK cells, NK T cells, and  $\gamma\delta$  T cells combined, all of which are innate immune cells.

The recently discovered innate lymphoid cells (ILCs) have been shown to have a role in the control of diseases. These cells produce many of the Th cell type cytokines but lack cell surface markers found on other immune cells, hence named lineage marker-negative (ILN), and they do not express a T cell receptor. The ILCs have been divided into three groups. The group which relates to this chapter are the ILC group 1. This encompasses the ILN-ILC1 and NK cells. The ILN-ILC1s express the surface marker CD56 and secrete IFN- $\gamma$  as it's signature cytokine (Reviewed in (Walker et al., 2013)). Thus these cells could be a key source of the IFN- $\gamma$  measured in CD56+ cells in this study. It will be interesting to apply additional markers in future studies to gate on the ILN-ILC1 subset for more indepth analysis.

Overnight stimulation with the vaccine antigens led to low-level cytokine production in CD56+ cells. In the DTwP group there was a decrease in CD56+IL-13+ cells in TT cultures post-vaccination which suggests a possible suppression of type 2 innate immunity. This effect was not apparent when MV and DTwP were administered at the same time, in support of altered responses when the live MV is given at the same time.

#### 6.4.4 Effects on Innate Responses to LPS, PPD and $\alpha$ CD3/ $\alpha$ CD28

Due to its broad specificity, reactivity to  $\alpha$ CD3/ $\alpha$ CD28 was generally about double that observed to the measles pool, TT or PPD. The only differential effect to  $\alpha$ CD3/ $\alpha$ CD28 stimulation was observed in the DTwP group, whereby males had significantly higher levels of CD56+IFN- $\gamma$ + cells compared to females post-vaccination; while females produced more of the regulatory cytokine IL-10 in CD56+ cells from PPD cultures, suggesting a pro-inflammatory innate effect in males and an immunosuppressive innate effect in females. It is possible that the  $\alpha$ CD3/ $\alpha$ CD28 effect is from NK T cells and  $\gamma\delta$  T cells within the CD56+ population since these would be stimulated by  $\alpha$ CD3/ $\alpha$ CD28, although this requires confirmation in future studies.

The LPS reactivity was higher than that observed to  $\alpha$ CD3/ $\alpha$ CD28, in particular IFN- $\gamma$ + CD56+ cells, which might be expected when conducting overnight cultures using whole blood in which the innate response is likely to be a major contributor. That NK cells respond to LPS (TLR4 agonist) is a fairly new discovery and Duriez et al has reported high IFN- $\gamma$  production by decidual NK cells (Duriez et al., 2014). The surface expression (O'Connor et al., 2006) as well as the functional activity of TLR4 (Mian et al., 2010) has been detected in human NK cells. A study by Kanevskiy and colleagues using LPS stimulation of purified TLR4<sup>+</sup>CD56+ NK cells followed by intracellular cytokine staining has shown a high production of IFN- $\gamma$  from these cells but not from other cell types, suggesting an alternative method of LPS signalling distinct from the known surface TLR4 mediated signalling (Kanevskiy et al., 2013). NK cells and DCs also express CD8 receptor and it is possible that LPS uses this receptor for signalling in these cells.

The increased Th2 (CD4+IL-13+) and decreased type 1 innate cell (CD56+IL-2+) responses in LPS cultures in the MV+DTP vaccinated group suggests a type 2 innate skewing following vaccination with MV and DTP simultaneously. By contrast, MV vaccinated males had greater Tc1 (CD8+IFN- $\gamma$ +) reactivity to LPS after vaccination

compared to the other male groups, supporting an innate bias towards type 1 immunity following MV in males but not females. The DTwP group males had more Th2 (CD4+IL-10+IL-13+) reactivity to LPS than females following vaccination. Thus innate type 1 skewing by MV may be lost when DTP is given with MV.

#### **6.4.5 Limitations**

There are a number of limitations that should be borne in mind when interpreting the results of this chapter. The percentages of the cells expressing the various cytokines were very low, although this is well recognised for this methodology and our data are comparable to most other studies using ICS methodology to detect cytokine producing cells (Sun et al., 1998; Godoy-Ramirez et al., 2004; Pahar et al., 2005; Van Hemelen et al., 2011; Yamashita et al., 2013). All cell subsets were detectable by flow cytometry, and were very low in the negative control samples, while our positive controls generated higher proportions of cytokine-producing cells. There were multiple cell subsets analysed for different culture conditions, with different vaccine groups compared both before and after vaccination and by sex, and thus the issue of multiple testing and false positive significant results increases. For this reason we have only reported significant results after correcting for FDR. Thus we are confident that those differences detected, whilst small, are likely to be meaningful.

We observed a number of pre-vaccination differences, making post-vaccination differences more difficult to interpret. Since the infants were 9 months old at baseline, their immune responses would be influenced by prior exposure to environmental stimuli, previous infections and vaccinations, maternal factors such as maternal antibodies and breast feeding, and genetics. Furthermore, the MV group had received 3 doses of DTwP while the other 2 groups had only received two, which could account for baseline differences between the MV group and the other 2 vaccine groups although some baseline differences were also observed exclusively in one of the other groups, likely to be due to a combination of the factors above.

#### 6.4.6 Overall conclusions

Despite the limitations above, vaccine group and sex differences were observed post-vaccination. Furthermore, the data suggest that co-administration of MV with DTP interferes with the priming of the measles specific response; and also leads to a different immune profile compared to when the vaccines are given alone, generally with decreased type 1 and enhanced type 2 responses. The putative immunosuppressive effects of DTwP suggested in the previous chapter are further supported by decreased CD4 reactivity in  $\alpha$ CD3/ $\alpha$ CD28 cultures after DTwP vaccination observed in this chapter.

Where male / female differences were observed, the males generally had stronger responses than females following MV or DTwP vaccination, while no sex differences were observed in the MV+DTwP group. This is consistent with the cytokine data from the previous chapter.

Therefore this chapter further characterizes vaccine group and sex differences that might account for the differential heterologous effects of the different vaccine schedules. The data are subject to many limitations and would require confirmation in further studies.

## **CHAPTER 7**

# **WHOLE HUMAN GENOME TRANSCRIPTOME PROFILING REVEALS SEX- DIFFERENTIAL EFFECTS FOLLOWING VACCINATION WITH DT<sub>w</sub>P AND MEASLES VACCINES**

## 7.1 Introduction

Conventional methods employed in immunological profiling of vaccine responses such as the enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunosorbent spot (ELISpot) assay, intracellular staining (ICS) by flow cytometry and cytokine analysis by multiplex technology, can only test a limited number of parameters, which is a particular problem in infants where small volumes of blood are available. The advent of systems biology technology, including transcriptome microarray, allows the rapid and simultaneous analysis of thousands of genes, and is beginning to provide new insights into the complex nature of the immune response to vaccines (Flanagan et al., 2013a).

As discussed throughout this thesis, there is increasing evidence for sex differences in immunity to vaccines, and our luminex and ICS data support this (Chapters 5 and 6 respectively). In a re-analysis of the data in the Querec paper (Querec et al., 2009), Klein and colleagues found that females differentially expressed many more innate immune response genes than males following yellow fever vaccination, with limited overlap in the genes differentially expressed in males and females (Klein et al., 2010). Data from Flanagan and colleagues replicates these findings in measles vaccinated infants (Flanagan et al, in preparation). Analysing males and females together may therefore lead to a loss of information since changes in gene expression may only become apparent when males and females are analysed separately.

Transcriptional profiling technology has not been used to study the heterologous or non-specific effects of vaccines to date. In this chapter, we therefore undertook whole human genome transcriptome profiling before and after vaccination in order to analyse for differential gene expression after vaccination in the three vaccine groups. In our previous chapters we have found sex differences for some of the parameters tested, and we therefore sought to analyse for sex differences in the transcription profile. In particular, we hoped to gain further insights into the nature of the immunosuppressive effects of DTwP in females, and the reversal in the MV+DTwP group of the normal pattern in which males

have stronger inflammatory responses than females. Overall, we hoped to use this technology to identify biomarkers / gene signatures that might be responsible for the beneficial and deleterious effects of vaccines, which might then be tested prospectively in future studies.



7.2 Methods

RNA was extracted at the MRC Unit, The Gambia from whole blood in PAXgene tubes from all available 9 and 10 month old study infant samples as detailed in Methods (chapter 2). RNA samples were sent to the Division of Pathway Medicine (DPM), University of Edinburgh for transcriptional profile analysis. Dr. Paul Dickinson at DPM, in partnership with Gen-Probe, Inc., performed the transcriptome analysis assays for this chapter. Dr. Paul Dickinson and Thorsten Forster at DPM, in partnership with Dr. Al Ivens, Fios Genomics Ltd., performed the statistical analysis for the transcriptome data. Please note that while I had the opportunity to visit Edinburgh and learn the technology, I did not carry out the assays or perform the analysis.

The samples available consisted of 202 donors with pre- and post-vaccination RNA samples available. Quality control using the Agilent Bioanalyser 2100 and Nanodrop ND1000 was used to determine those RNA samples of sufficient quality and quantity and 360 samples were selected for subsequent microarray analysis, of which 348 samples contributed data to the final dataset (192 female samples and 196 male samples) (Table 7.1). The Illumina HT12v4 Array containing 47,293 array probes was used for the analysis as detailed in Methods (chapter 2). Linear modelling was used to explore the data after which empirical Bayesian analysis was applied (including vertical (within a given comparison) p value adjustment for multiple testing, which controls for false discovery rate). The Bioconductor package limma was used (Smyth et al., 2005)). Multiple samples from the same individual were taken into account when modelling the data.

Table 7.1 Number of donors analyzed by group and sex

|         | Males<br>Unpaired |      | Males<br>Paired |      | Females<br>Unpaired |      | Females<br>Paired |      |
|---------|-------------------|------|-----------------|------|---------------------|------|-------------------|------|
|         | Pre               | Post | Pre             | Post | Pre                 | Post | Pre               | Post |
| MV      | 32                | 31   | 31              | 31   | 26                  | 27   | 24                | 24   |
| MV+DTwP | 30                | 31   | 29              | 29   | 35                  | 35   | 32                | 32   |
| DTwP    | 26                | 26   | 26              | 26   | 25                  | 24   | 23                | 23   |
| Total   | 88                | 88   | 86              | 86   | 86                  | 86   | 79                | 79   |

*The number of donors analyzed for each vaccine group for each sex in the paired and unpaired analysis.*

100ng total RNA was converted to double-stranded cDNA, followed by an amplification step (*in vitro* transcription) to generate labelled cRNA, using the Ambion Illumina TotalPrep-96 RNA Amplification Kit. This produced an amplified pool of biotin-labelled cRNA corresponding to the polyadenylated (mRNA) fraction. The cRNA was quantified using the Nanodrop ND1000, cRNA quantity was normalised and then hybridised onto the Illumina HT12 arrays for 14-20 hours at 58°C. The unhybridized and non-specifically hybridized cRNA was washed away and arrays stained with Cy3-Streptavidin to bind to the analytical probes hybridized to the array and allow differential detection of signals when the arrays were scanned.

Arrays were scanned with an Illumina IScan two-color laser scanner (532 nm/658 nm) with a 0.53  $\mu$ m spatial resolution capable of exciting the fluorophores generated during the staining step of the protocol. Light emissions from these fluors were then recorded in high-resolution images of the arrays. Intensities of the images were extracted using GenomeStudio (2010.3) Gene Expression Module (1.8.0) software. Normalization of data and statistical tests are detailed in Methods (chapter 2).

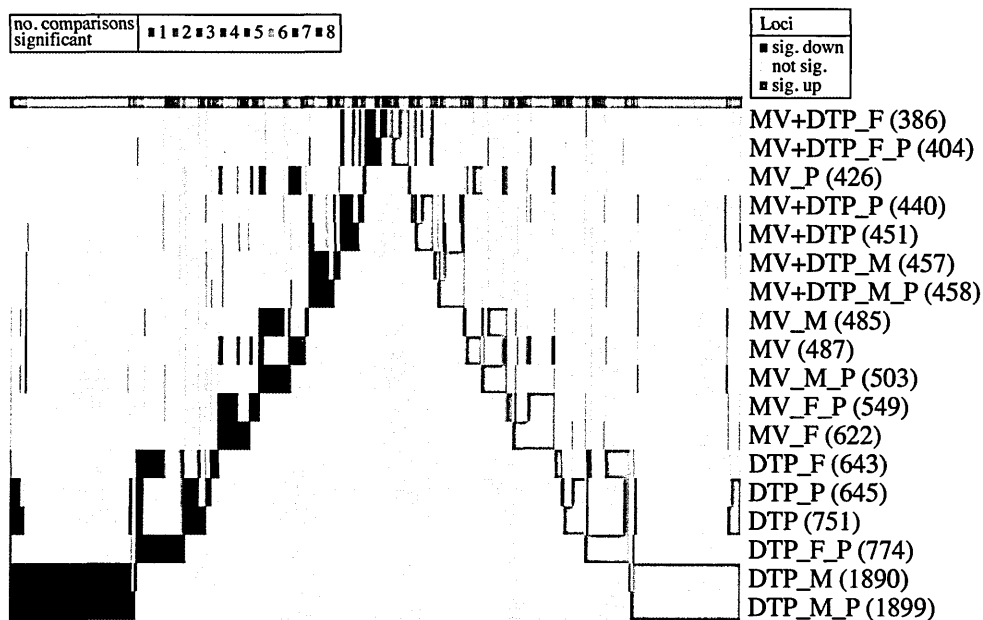
To identify differentially expressed genes post-vaccination relative to pre-vaccination in the three vaccine groups stratified according to sex, statistical analysis using linear model fitting and an empirical Bayes approach was used as detailed in Methods (chapter 2). Multiple testing correction was applied to the statistical testing using Benjamini-Hochberg p value adjustment for multiple testing implemented in the limma software package for R, which controls for false discovery rate. Expression networks were constructed employing the Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, [www.ingenuity.com](http://www.ingenuity.com)). Illumina Probe IDs were imported into the Ingenuity software and mapped to the Gene Symbol from the Ingenuity database as detailed in Methods (chapter 2).

## 7.3 Results

### 7.3.1 Divergent gene expression profiles is revealed in females and males depending on vaccination group

A total of 5,915 non-redundant array features were significant at raw  $p < 0.01$  (unadjusted) in one or more of the comparisons as detailed in Methods (chapter 2). Figure 7.1 shows the non-redundant set of 5,915 significant genes (X-axis) broken down by comparisons (Y-axis), with the number of significant genes for each comparison shown in parentheses after the comparison description. Upregulated genes are shown in red, down-regulated genes in blue; while loci that were not significant in a given comparison are shown in grey. Comparisons with no significant genes were removed. The comparisons are ordered in the plot such that the one with the highest number of significant changes is shown at the bottom. The significance landscape view of the data provides a direct visual representation of which array features were significant in which comparison, thus enabling "condition" specific and/or enriched loci to be rapidly identified.

Statistical profiles for each of the comparisons were obtained. For each comparison, the number of array features significant at various statistical thresholds were tallied but for statistical robustness, only those with an adjusted  $p$  value  $< 0.05$  were considered (Table 7.2).



**Figure 7.1** Significance landscape of differentially expressed probes. The non-redundant set of 5,915 significant genes (X-axis), by comparisons (Y-axis; the number of significant genes in each comparison are shown in parentheses after the comparison description); comparisons with the highest number of significant changes are shown at the bottom. Red = upregulated, blue = down-regulated, grey = non-significant in a given comparison.

**Table 7.2** Array features significant at various statistical thresholds

| Index | Contrast                         | rawP<0.05 | rawP<0.01 | rawP<0.001 | rawP<0.0001 | adjP<0.05 | adjP<0.01 | adjP<0.001 | adjP<0.0001 | B>=0 |
|-------|----------------------------------|-----------|-----------|------------|-------------|-----------|-----------|------------|-------------|------|
| 17    | MV post_pre                      | 2294      | 486       | 42         | 3           | 0         | 0         | 0          | 0           | 7    |
| 18    | DTP post_pre                     | 3409      | 748       | 89         | 12          | 0         | 0         | 0          | 0           | 29   |
| 19    | MV+DTP post_pre                  | 2233      | 448       | 44         | 5           | 0         | 0         | 0          | 0           | 11   |
| 1     | MV F post_pre                    | 2627      | 619       | 80         | 17          | 1         | 0         | 0          | 0           | 19   |
| 2     | MV M post_pre                    | 2544      | 485       | 53         | 8           | 0         | 0         | 0          | 0           | 6    |
| 3     | DTP F post_pre                   | 2856      | 642       | 86         | 10          | 0         | 0         | 0          | 0           | 14   |
| 4     | DTP M post_pre                   | 5998      | 1889      | 250        | 22          | 0         | 0         | 0          | 0           | 86   |
| 5     | MV+DTP F post_pre                | 1910      | 385       | 35         | 3           | 0         | 0         | 0          | 0           | 3    |
| 6     | MV+DTP M post_pre                | 2390      | 457       | 52         | 5           | 0         | 0         | 0          | 0           | 3    |
| 21    | MV post_pre_paired               | 2182      | 425       | 31         | 3           | 0         | 0         | 0          | 0           | 5    |
| 22    | DTP post_pre_paired              | 2967      | 642       | 82         | 10          | 0         | 0         | 0          | 0           | 21   |
| 23    | MV+DTP post_pre_paired           | 2168      | 440       | 51         | 7           | 0         | 0         | 0          | 0           | 11   |
| 9     | MV F post_pre_paired             | 2424      | 548       | 59         | 18          | 1         | 1         | 0          | 0           | 15   |
| 10    | MV M post_pre_paired             | 2796      | 501       | 51         | 7           | 0         | 0         | 0          | 0           | 4    |
| 11    | DTP F post_pre_paired            | 3157      | 773       | 89         | 9           | 0         | 0         | 0          | 0           | 25   |
| 12    | DTP M post_pre_paired            | 5994      | 1895      | 244        | 19          | 0         | 0         | 0          | 0           | 85   |
| 13    | MVDTP F post_pre_paired          | 1944      | 404       | 51         | 2           | 0         | 0         | 0          | 0           | 2    |
| 14    | MVDTP M post_pre_paired          | 2410      | 458       | 56         | 7           | 0         | 0         | 0          | 0           | 4    |
| -     | Non-redundant markerset (nr set) | 19512     | 5915      | 779        | 99          | 1         | 1         | 0          | 0           | 201  |

The number of array features significant at various statistical thresholds for each comparison post- relative to pre-vaccination for paired and unpaired data. Probesets with  $B \geq 0$  are more likely to be differentially expressed than not. F = Females, M = males where the data is analyzed by sex. DTWP is shortened to DTP for lack of space.

After multiple test adjustment (corrected using Benjamini & Hochberg method for multiple testing implemented in the limma software package for R) only 2 comparisons showed any statistically significant genes (MV females paired and unpaired – 1 probe only). In light of this and in order to attempt to understand the nature of any transcriptional changes between vaccine treatments it was felt that for this dataset a less stringent approach was



### 7.3.2 Distinct group and sex differences in differentially expressed genes

There was little differential gene expression greater than 1.5 fold unless the vaccine groups were separated by sex. The majority of non-redundant differentially expressed probes occurred in MV+DTwP males and DTwP males and females post-vaccination compared to pre-vaccination. A list of differentially expressed genes was derived from the above analyses (Table 7.3) and uploaded into the Ingenuity Pathway Analysis software to examine network and functional relationships between genes differentially expressed following vaccination with the 3 different schedules. However, the numbers of differentially expressed genes in MV vaccinated females and males, and MV+DTwP vaccinated females were too small for effective pathway analysis, thus networks were only generated for MV+DTwP males and DTwP vaccinated males and females.

Although the number of genes in MV and MV+DTwP females was too small for pathway analysis, several probes were differentially expressed in both groups including interferon inducible gene 27 (IFI27) which was upregulated in both groups (Table 7.3). TXNDC5 differentially expressed in MV+DTwP females was upregulated and has a role in protecting hypoxic cells from apoptosis. Only one gene (DEFA3) was differentially expressed in MV males. This upregulated gene codes for a defensin, which has microbicidal and cytotoxic functions, which would aid in defense against invading pathogens. When examining the numbers of individuals showing a greater than 1.5 fold differential response for all of the differentially expressed probes (Table 7.3) it is clear that there is heterogeneity in the response, whereby some probes are highly differential in some individuals while not so in others. However, most of the changes are based upon a response in a number of individuals often amounting to half of the study group and not just due to a large response in one or two individuals.

**Table 7.3 Genes present in non-redundant probe list in the three vaccine groups by sex**

| Vaccine Group / Sex | Non-redundant probes (paired and unpaired)  |
|---------------------|---|
| MV F                | IFI27 (10/24), PROK2 (10/24), RPL7 (14/24)  |
| MV M                | DEFA3 (12/31)   |
| MV+DTwP F           | IFI27 (19/32), IGJ (18/32), OTOF (14/32), TXNDC5 (17/32)  |
| MV+DTwP M           | ADRA2C (13/39), ARTN (13/39), C1orf150 (3/29), CSDAP1 (14/29), DPYSL5 (13/29), GATSL3 (13/29), LOC100131391 (4/29), LOC100131726 (13/29), LOC440313 (13/29), LOC642469 (14/29), MUC6 (13/29), IGFBP1 (15/29), RNF213 (13/29), SERPINA13 (13/29), SMOX* (15/29), SMOX* (15/30), SPRYD3 (13/29), TPRA1 (13/29)  |
| DTwP F              | EPSTI1 (10/22), FCGR3B (11/22), GBP1 (10/22), GBP1 (10/22), HERC5 (11/22), IFI44 (10/22), IFI44L (10/22), IFI6 (8/22), IFIH1 (8/22), IFIT1 (10/22), IFIT2 (14/22), IFIT3* (10/22), IFIT3* (10/22), IFIT3* (10/22), IFITM3 (11/22), IL1RN (9/22), ISG15 (10/22), LAP3 (10/22), MX1 (10/22), MARCKS (10/22), OAS1* (10/22), OAS1* (10/22), OAS3 (10/22), OASL (10/22), RSAD2 (10/22), SERPING1 (10/22), SPATS2L (10/22), STX11 (10/22), TRIM22 (8/22) |
| DTwP M              | ABHD3 (14/26), ADD3 (11/26), BNIP3L (12/26), CLK1 (11/26), DR1 (16/26), Hs.657419 (12/26), JMJD1C (13/26), MBNL1 (10/26), MST4 (9/26), OSBPL8 (2/26), PCMTD1 (12/26), PRNP (11/26), RASGRP1 (11/26), SENP7 (9/26), TMED5 (11/26), ZFAND5 (11/26)  |

*Genes  $\geq 1.5$  fold expression present in non-redundant probe lists following unpaired and paired analyses. \* Indicates multiple probes for the same gene represented. Red text = upregulated genes and blue text = down-regulated genes. Numbers in parentheses represent the number of individuals that changes of at least 1.5 fold were found in divided by the number of individuals in the study group.*

**7.3.3 DTwP vaccinated females have down-regulated innate and antiviral gene pathways**

DTwP vaccinated females showed down-regulation of genes encoding inflammatory and antiviral factors after vaccination, with 29 probes showing greater than 1.5 fold differential expression (Table 7.3). When these 29 probes were uploaded to Ingenuity Pathway Analysis (IPA) software, 25 genes were analysis ready due to the presence of duplicate probes. Allowing networks of up to 70 components created by direct interaction, one major network was produced (Network score 67) containing all 25 focus molecules. Top

canonical pathway associated functions as revealed by network analysis include interferon signaling and dendritic cell (DC) maturation (Table 7.4; Figure 7.3).

Many of the down-regulated genes are well-known type 1 interferon stimulated genes (ISGs), including IRF7, IFIT1, IFIT2, IFIT3, IFITM3, IL1RN, ISG15, MX1, OAS1, OAS3, OASL, RSAD2. Most of them are commonly induced by viral infection and play essential roles in type 1 IFN signaling, antigen presentation, and cellular and humoral immune responses (Table 7.4). For example, the down-regulated radical S-adenosyl methionine domain containing 2 (RSAD2) gene plays a key role in anti-viral immunity by promoting TLR7 and TLR9 dependent production of IFN- $\gamma$  by pDCs, and also plays a role in CD4<sup>+</sup> T cell activation and differentiation, in particular optimal Th2 cytokine production. The ubiquitin-like modifier (ISG15) gene is involved in the viral sensing RIG-I-like receptor signaling pathway as well as interferon signaling and anti-viral immunity. ISG15 also activates neutrophils to produce eosinophil chemotactic factors. HECT and RLD domain containing E3 ubiquitin protein ligase 5 (HERC5), which has a positive regulatory role in the innate antiviral response in cells induced by interferon, was also down-regulated after DTwP vaccination of females (Table 7.4). Another interesting down-regulated gene was interleukin 1 receptor antagonist (IL1RN), which plays a role in acute phase signaling and cell mediated immunity by inhibiting the inflammatory action of IL-1 $\alpha$  and IL-1 $\beta$ .



**Table 7.4 Pathway description and function of differentially expressed genes in DTwP vaccinated females**

| Symbol | Pathway Description  | Gene Name  | Function  | Fold change |
|--------|--|--|---|-------------|
| IFIT2  | Interferon signaling   | Interferon-induced protein with tetratricopeptide repeats 2  | Cell-mediated immune response, Antiviral activity       | -2.297      |
| RSAD2  | Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses | Radical S-adenosyl methionine domain containing 2            | Cell-mediated immune response, Response to virus        | -2.144      |
| IFIT1  | Interferon Signaling   | Interferon-induced protein with tetratricopeptide repeats 1  | Molecular Transport, Antiviral defense, Innate immunity | -2.000      |
| IFIT3  | Interferon signaling   | Interferon-induced protein with tetratricopeptide repeats 3  | Antiviral defense, Innate immunity                      | -1.986      |
| HERC5  | Protein modification; protein ubiquitination                                 | HECT and RLD domain containing E3 ubiquitin protein ligase 5 | Post-Translational Modification                         | -1.945      |
| IFI44  | Activation of IRF by Cytosolic Pattern Recognition Receptors                 | Interferon-induced protein 44                                | Response to virus                                       | -1.905      |
| ISG15  | RIG-I-like receptor signaling pathway; Interferon signaling                  | ISG15 ubiquitin-like modifier                                | Antiviral response                                      | -1.840      |
| OAS3   | Interferon signaling   | 2'-5'-oligoadenylate synthetase 3                            | Antiviral response                                      | -1.828      |
| IFITM3 | Interferon signaling   | Interferon induced transmembrane protein 3                   | Response to virus                                       | -1.828      |
| IFI44L | Interferon signaling   | Interferon-induced protein 44-like                           | Immune response; defense response to virus              | -1.828      |
| IL1RN  | Acute Phase Response Signaling   | Interleukin 1 receptor antagonist                            | Lipid Metabolism; Cell-mediated Immune Response         | -1.516      |

*Description and functions assigned to the networks of genes that were differentially expressed using Ingenuity Pathway Analysis (IPA) software in DTwP vaccinated female infants. The top 10 differentially expressed genes were select.*



transcriptional modification pathways (Table 7.5, Figure 7.4). The top canonical pathway associated functions were assembly of RNA polymerase II complex, T-cell receptor signaling and protein kinase A signaling. The major network associated functions mainly revealed an up-regulation of genes that are involved in developmental and repair functions.

Two apoptosis pathway genes were upregulated, namely BCL2/adenovirus E1B 19kDa interacting protein 3-like (BNIP3L) and serine/threonine protein kinase (MST4); while the upregulated prion protein (PRNP) gene plays a role in cell death and survival. The zinc finger, AN1-type domain 5 (ZFAND5) gene which is involved in both the TNF-alpha / NF-kB signaling pathways and Forkhead box O (FoxO) family signaling was upregulated following vaccination. The FoxO family genes regulate genes of cell growth, differentiation and proliferation. The adducin 3 gene (ADD3), jumonji domain containing 1C (JMJD1C) and RAS guanyl-releasing protein 1 (RASGRP1) genes are all involved in hematological system development. Only one upregulated gene - down-regulator of transcription 1 (DR1), is involved in transcription processes.

**Table 7.5 Pathway description and function of differentially expressed genes in DTwP vaccinated males**

| Symbol  | Pathway Description                                      | Gene Name  | Function  | Fold change |
|---------|--|--|---|-------------|
| ADD3    | Activation of cAMP-Dependent PKA                         | Adducin 3 (gamma)  | Hematological System Development and Function; Tissue Morphology    | 1.625       |
| JMJD1C  | Leukocyte Extravasation Signaling                        | Jumonji domain containing 1C   | Hematopoiesis   | 1.580       |
| MST4    | Apoptotic pathway  | Serine/threonine protein kinase MST4                                 | Cellular Growth and Proliferation                                   | 1.580       |
| ANKRD10 | N/A  | ankyrin repeat domain 10   | N/A   | 1.558       |
| ZFAND5  | TNF-alpha/NF-kB Signaling Pathway; FoxO family signaling | Zinc finger, AN1-type domain 5                                       | N/A   | 1.558       |
| DR1     | Chromatin Regulation / Acetylation                       | Down-regulator of transcription 1, TBP-binding (negative cofactor 2) | Assembly of RNA Polymerase II Complex                               | 1.548       |
| OSBPL8  | N/A  | Oxysterol binding protein-like 8                                     | N/A   | 1.548       |
| RASGRP1 | Gas Signaling, T cell receptor signaling pathway         | RAS guanyl releasing protein 1 (calcium and DAG-regulated)           | Hematological System Development and Function; Cellular Development | 1.548       |
| PRNP    | Prion diseases   | Prion protein  | Cellular Development; Cell Death and Survival                       | 1.537       |
| BNIP3L  | Apoptosis  | BCL2/adenovirus E1B 19kDa interacting protein 3-like                 | Cancer; Induces apoptosis   | 1.526       |

*Description and functions assigned to the networks of genes that were differentially expressed using Ingenuity Pathway Analysis (IPA) software in DTwP vaccinated male infants. The top 10 differentially expressed genes were select.*



**Table 7.6 Pathway description and function of differentially expressed genes in MV+DTwP vaccinated males**

| Symbol | Pathway Description  | Gene Name                                     | Function  | Fold change |
|--------|--|---|---|-------------|
| SMOX   | Amine and polyamine degradation; spermine degradation              | Spermine oxidase                              | Regulation of polyamine intracellular concentration                 | -1.647      |
| DPYSL5 | Axon guidance  | Dihydropyrimidinase-like 5                    | Neural development  | -1.636      |
| DVL1   | Wnt signaling pathway, Notch signaling pathway, Pathways in cancer | Dishevelled, dsh homolog 1 (Drosophila)       | Regulation of cell proliferation, Cancer, Dermatological Conditions | -1.613      |
| RNF213 | Protein modification; protein ubiquitination.                      | Ring finger protein 213                       | Ubiquitin-protein ligase activity                                   | -1.580      |
| SPRYD3 | N/A  | SPRY domain containing 3                      | N/A   | -1.548      |
| TPRA1  | G-protein coupled receptor signaling pathway                       | Transmembrane protein, adipocyte associated 1 | Small Molecule Biochemistry   | -1.537      |
| MUC6   | N/A  | Mucin 6, oligomeric mucus/gel-forming         | Tissue Development  | -1.526      |
| ARTN   | N/A  | Artemin                                       | Cellular growth and proliferation                                   | -1.516      |
| ADRA2C | Neuroactive ligand-receptor interaction                            | Adrenergic, alpha-2C, receptor                | Nervous system development and function                             | -1.516      |
| IGFBP1 | Insulin receptor signaling pathway                                 | Insulin-like growth factor binding protein 1  | Cellular growth and proliferation                                   | -1.505      |

*Description and functions assigned to the networks of genes that were differentially expressed using Ingenuity Pathway Analysis (IPA) software in MV+DTwP vaccinated male infants. The top 10 differentially expressed genes were select.*

Genes involved in protein and amino acid metabolism include spermine oxidase (SMOX) and ring finger protein 213 (RNF213) (Table 7.6). Artemin (ARTN) and insulin-like growth factor binding protein 1 (IGFBP1) were down-regulated, both of which promote cellular growth and proliferation; as was the disheveled segment polarity protein 1 (DVL1) gene which is a regulator of cell proliferation. Two of the genes, dihydropyrimidinase-like 5 (DPYSL5) and adrenergic, alpha-2C, receptor (ADRA2C), are important in nervous system development and function. The spry domain containing 3 (SRPYD3) gene is associated with agammaglobulinaemia suggesting a role in IgG production; and the mucin 6 (MUC6)



## 7.4 Discussion

We aimed to use transcriptome analysis to help identify signatures that might account for beneficial or deleterious effects of vaccines, and further analyse for key sex differences. It is apparent using this approach that females and males in the different vaccine groups have distinct transcriptional profiles 4 weeks after vaccination. It should be borne in mind that the 4 week time point is not an ideal time point at which to analyze for transcriptional changes post-vaccination with very few statistically significant differences observed between any of the pre- and post-vaccination groups following false discovery correction. Differential gene expression was weak and we had to use less stringent analysis criteria to tease out group and sex differences than we had intended. Data published after the study was designed show that early signatures from 1-7 days post-vaccination predict vaccine responses, and might thus have been more informative. Indeed, in a time course transcriptome profiling study carried out in the same study site in The Gambia, responses to measles vaccination of 9 month old infants peaked at 1 week and had declined by 4 weeks (Flanagan et al, in preparation). However, it was not possible to collect an extra early blood sample for logistic reasons, and furthermore we were more interested in non-specific effects of vaccination, which are thought to last for months after the vaccine is given.

### 7.4.1 Down-regulated type 1 IFN pathways and DC function could explain deleterious effects of DTwP vaccination among female infants

One major finding in this study was the marked down-regulation of interferon response pathways in females 4 weeks after vaccination with DTwP. Type 1 interferons are very important in the control of viral infections in particular, but also certain intracellular bacteria such as *Mycobacterium tuberculosis*. Type1 IFNs and type1 interferon stimulated genes (ISGs) play a significant role in the innate immune response, but can also modulate the type and magnitude of the subsequent adaptive immune response via dendritic cells (Stark et al., 1998; Santini et al., 2002; Tough, 2004; Kawada et al., 2006; Steinman and Hemmi, 2006; Katze et al., 2008; Proud et al., 2008; Yao et al., 2009). A sustained or delayed



down-regulation of type 1 IFN response pathway in DTwP vaccinated females following vaccination could be a possible explanation for the non-specific effects of DTwP vaccination of females (Aaby et al., 2003c; Aaby et al., 2007; Benn et al., 2009a; Benn et al., 2010; Roth et al., 2010). Again, this is speculative and would need to be validated in future experiments. Such a down-regulation would be hypothesised to lead to an increased susceptibility to viral infections.

These data fit with the protein data in which we saw immunosuppression for multiple parameters in DTwP vaccinated females. DTwP vaccinated females had suppressed response to PPD stimulation with suppressed CD8 (Tc1) T cell reactivity by ICS. They also had broadly suppressed reactivity to  $\alpha$ CD3/ $\alpha$ CD28 stimulation with suppressed IFN- $\gamma$ /IL-10 ratio in culture supernatants. The DTwP vaccinated group in general had suppressed CD4 (IFN- $\gamma$ ) and CD8 (IFN- $\gamma$ ) T cell reactivity to  $\alpha$ CD3/ $\alpha$ CD28 and LPS stimulations respectively by ICS. These would need to be validated in future studies.

Children vaccinated with live attenuated influenza vaccine (LAIV) had higher differential expression of type 1 IFN and ISGs, but this was not the case in the study group vaccinated with trivalent inactivated influenza vaccine (TIV) (Zhu et al., 2010b). This would suggest that live vaccines are more likely to stimulate type 1 IFN genes than inactivated vaccines. However, the authors do not describe a down-regulation of type 1 IFN genes following TIV vaccination. Since we only have the 4 week post-vaccination time point we do not know if these genes are up-regulated at any point following DTwP vaccination in females, and this will remain the work of future studies.

#### **7.4.2 Upregulated genes involved in cell development and repair in DTwP vaccinated males**

In contrast to the generalized down-regulation of genes in DTwP vaccinated females, the DTwP vaccinated males had upregulated genes only. Furthermore, the upregulated genes

were completely different to those down-regulated in the females, with the major pathways upregulated being those involved in development and repair.

There was upregulation of several genes involved in apoptosis and cell survival in DTwP vaccinated males. The BNIP-3L gene protects cells from viral-induced cell death, while MST4 is thought to play a role in the apoptotic pathway. The upregulation of the ZFAND5 gene, involved in the regulation of TNF- $\alpha$  / NF- $\kappa$ B signaling pathways, may also play a role in apoptosis. It would therefore be of interest to study the effect of DTwP vaccination on immune cell apoptosis and cell death in males and females.

The down-regulator of transcription 1 (DR1) gene was upregulated after vaccination. This gene encodes a protein that represses and controls the rate of RNA polymerase II transcription. A recent study showed that OSBPL8 is important in the interferon antiviral response. The mechanism of this antiviral response involves the recruitment of STAT1 to the promoter region of the ch25h gene resulting in the transcription and translation of a single oxysterol (25HC) by macrophages (Blanc et al., 2013). Other studies report the role of OSBPL8, as a negative regulator of cholesterol efflux from macrophages (Yan et al., 2008). This would therefore suggest that DTwP vaccination may affect macrophage function in males.

There were several upregulated genes that are involved in haematological functions in DTwP vaccinated males. The ADD3 gene plays an important role in hematological system development and function, and the JMJD1C gene is involved in hematopoiesis. The upregulated RASGRP1 gene activates the Erk/MAP kinase cascade and regulates T and B cell development, homeostasis and differentiation. An upregulation of such functions would be hypothesised to be beneficial to the host.

Thus DTwP vaccinated males would be hypothesised to have better cellular repair processes, less apoptosis and improved cell survival following vaccination than DTwP

vaccinated females. They may also have improved anti-viral IFN responses via enhanced macrophage function. This would be in keeping with the enhanced pro-inflammatory innate and T cell functions in males compared to females in the cytokine and ICS studies. These putative effects would need to be verified in more detailed functional studies, but would presumably be beneficial to the infant.

It is interesting that the genes that were differentially expressed in DTwP vaccinated females were all downregulated and are quite different to the upregulated genes in DTwP vaccinated males. This would be hypothesised to be due to sex differences. It is important to note that the microarray analysis is only indicative of changes that may occur upon vaccination and can be used to direct further work to validate these potential alterations. We would like to stress that these results are preliminary and would need to be followed up with validation experiments.

#### **7.4.3 Negative transcription effects in MV+DTwP vaccinated males**

In contrast to the DTwP vaccinated males, co-administration of MV with DTwP resulted in down-regulated developmental pathways in males, particularly of genes involved in amino acid metabolism and signaling processes. Several of the down-regulated genes promote cell growth and proliferation, and thus one would predict negative effects of this gene down-regulation. The down-regulated MUC6 gene might lead to less protected epithelial barriers against pathogen challenge.

This suggests that combining the MV and DTwP vaccines may cause a switch from generalized gene upregulation in DTwP vaccinated males, to down-regulation when both vaccines were administered together. Overall we would hypothesise that this would have negative effects in these males.

#### 7.4.4. Limitations

This study had several limitations that should be noted. Samples were collected at a single time point 4 weeks after vaccination and the dynamics of the immune response in males and females may be different. We had to use less stringent analyses to enable us to identify differential expression and these data are also preliminary and require validation, therefore the transcriptome data should be interpreted with caution.

#### 7.4.5 Overall Conclusions

There are quite diverse genes and pathways involved in mediating immunologic memory and these can change quickly. Measuring multiple genes and pathways over time using microarray analysis is therefore helpful in understanding the immune response to vaccination. It provides a broad overview of the multitude of immune pathways affected by vaccination, and when used in conjunction with other assays that measure antibody and cellular responses, it provides an in-depth knowledge of innate and adaptive responses to vaccines. This may in turn allow identification of predictors of vaccine efficacy, vaccine reactogenicity and non-targeted effects of vaccines on the immune system.

Our data contribute to our understanding of the immunological effects of measles and DTwP vaccination of infants, and the effect of administering the two vaccines at the same time. In keeping with the protein data in the previous 2 chapters, there were distinct differences both between vaccine groups and sexes. Furthermore, the RNA data gave further indications as to the nature of some of the observed protein effects. The down-regulated innate / type 1 IFN pathways in DTwP vaccinated females is in concordance with the immunosuppressive effects post-vaccination *in vitro*. By contrast, the upregulated genes in DTwP males corresponds with the more pro-inflammatory profiles in this group *in vitro*. When MV was given with DTwP the males switched to a down-regulation of genes, which fits with the protein data which shows that males become less inflammatory when both vaccines are given together. These results will however need validation since these results are preliminary.

The down-regulation of the type 1 IFN-stimulated genes might serve as a potential biomarker for the deleterious effects of DTwP in females if validated. We were unable to investigate the dynamics of this RNA response or whether innate genes are up-regulated at any point following vaccination in this study. However, the fact that they are down-regulated 4 weeks after vaccination could mean that the innate response would be weaker in these females thus increasing their susceptibility to infectious challenge. This is of particular importance in early life when the child is more reliant on the innate immune system for protection against invading organisms. This poorer innate immune response would be hypothesised to affect the development of the subsequent adaptive response, since they are intrinsically linked.

These data can now be used to select candidate genes or pathways that might account for some of the sex differences and non-specific effects of vaccines. These candidate genes can be selected alongside the protein data in order to determine biomarkers or signatures that might predict the beneficial or non-specific effects of vaccines. This is the subject of ongoing studies.

**CHAPTER 8**  
**CONCLUDING REMARKS**

## 8.1 Final conclusions

The expanded programme of immunization (EPI) was introduced in The Gambia over 30 years ago, using empirically developed vaccines with limited understanding of exactly how they work. Observational studies showing vaccines may have heterologous beneficial or deleterious effects on disease susceptibility, dependent on sex, have been controversial, with no immunological data to support these epidemiological findings (Aaby et al., 1995; Aaby et al., 2003c; Aaby et al., 2004b; Aaby et al., 2009; Agergaard et al., 2011; Aaby and Benn, 2011; Benn and Aaby, 2012).

In 2012 the Strategic Advisory Group of Experts on immunization (SAGE) at WHO commissioned a review of the evidence for the non-specific effects of vaccines. Two independent systematic reviews were conducted of the epidemiological and the immunological evidence respectively, focusing on BCG, DTP and measles vaccines in under 5 year olds. While recognising the plausibility of heterologous immunological effects of vaccines, they concluded that the existing data neither excluded nor confirmed a deleterious heterologous effect of DTwP on all-cause mortality; but acknowledged a possible beneficial heterologous effect of MV on all-cause mortality ((WHO), 2014). The expert group emphasised the need for further randomized trials with sufficient power to explore sex differences, and the need to explore and understand the immunological mechanisms.

This study is the first longitudinal randomized trial investigating the immunological consequences of vaccination with a live (Measles vaccine (MV)) or killed (Diphtheria, Tetanus, whole-cell Pertussis (DTwP)) vaccine or a combination of the two, with the aim of determining whether these vaccines have heterologous or non-specific effects which differ in males and females. Few studies have assessed antigen specific cytokine responses to vaccines in humans, but changes in the type-1 and type-2 immune profile would be expected to affect downstream immune responses. Therefore, a major aim of this thesis was to assess the effect of vaccination of cytokine profiles.

This study provides evidence for different immunological responses depending on the vaccine schedule and sex of the infant. We show evidence for sex-differential effects on immune responses to non-vaccine recall and innate antigens, which could potentially account for some of the previous epidemiological observations. These will need further analyses in carefully designed prospective studies.

#### **8.1.1 Vaccine-specific effects**

IgG antibody levels to all vaccines were comparable in males and females and importantly, were not affected when MV and DTwP were administered at the same time indicating that different schedules still resulted in protection to the different vaccines, at least in terms of antibody levels.

Cytokine profiles following culture with measles peptides showed a type 1 cytokine bias in measles vaccinated infants, and ICS results suggested a CD8 T cell source of the IFN- $\gamma$ . There were certain differences in measles pool cytokine reactivity dependent on the sex of the infant both before and after vaccination. After vaccination, males had higher IFN- $\gamma$  responses to measles peptides than females in the MV group.

Although protective antibody levels were induced to DTwP vaccination, cellular responses to TT antigen were very low in the DTwP vaccinated groups. However, TT responses were boosted in the MV+DTwP and DTwP groups, although more so in the MV+DTwP group. This suggests that MV has an adjuvant effect on DTwP responses with enhanced TT-specific immunity. The adjuvant effect by MV would be in keeping with the adjuvant effect provided by another live vaccine, BCG (Ota et al., 2002; Ritz et al., 2013). One could hypothesise that these enhanced immune responses would be beneficial in terms of pathogen clearance, but alternatively might lead to excessive inflammation and poorer outcomes, but again, this is speculative and will need to be investigated in future trials.



These effects on vaccine specific responses add novel data to our current understanding of the immunological effects of MV and DTwP vaccination.

### **8.1.2 Non-specific effects on responses to $\alpha$ CD3/ $\alpha$ CD28 and PPD stimulation**

Responses following PPD and  $\alpha$ CD3/ $\alpha$ CD28 stimulation allowed us to determine whether different vaccine schedules impacted on cellular responses to non-vaccine related stimuli. One common feature was an immunosuppressive effect of DTwP, particularly in females. The combined vaccine group generally behaved differently to the single vaccine groups in terms of male/female differences with males in both the MV and DTwP groups having greater pro-inflammatory responses to PPD and  $\alpha$ CD3/ $\alpha$ CD28 than females; while in the MV+DTwP group males and females generally had comparable responses or females has higher reactivity.

### **8.1.3 Effects on RNA transcription**

Differential gene expression following vaccination was extremely low level and the stringency of the analysis had to be decreased in order to identify sex and group differences. Since the samples were taken 4 weeks after vaccination and analysed for differential gene expression from whole unstimulated blood then this is not unexpected, and perhaps it is more surprising that any differences emerged at all. No group differences were observed unless the data were analyzed separately by sex. There was evidence of a down-regulation of interferon response pathways in females four weeks after vaccination with DTwP, further supporting an immunosuppressive effect of DTwP vaccination in females. The down-regulated genes were mainly type 1 interferon induced genes which are crucial to the innate control of many infections.

In contrast all the genes differentially expressed 4-week after DTwP vaccination in males were up-regulated, particularly genes involved in developmental pathways. Interestingly, four weeks after MV+DTwP, males had a quite different profile with all differentially expressed genes being down regulated. These data are consistent with the protein data in

which we observed a more pro-inflammatory profile in DTwP males compared to females, but a reversal or loss of this difference in the MV+DTwP group.

## **8.2 A putative model to account for the beneficial effects of MV and deleterious effects of DTwP vaccine**

Bearing in mind the exploratory nature of the results in this thesis and the need for further validation, we propose a model of heterologous effects of MV and DTwP whereby:

- (1) A less robust innate and T cell immunity in measles vaccinated females is beneficial to them.
- (2) DTwP causes a downregulation of innate gene transcription and suppression of innate and T cell immunity in females which increases their susceptibility to infections.
- (3) Co-administration of DTwP with MV causes females to become more pro-inflammatory, reducing the MV benefits to females; while dampening the immunosuppressive effects of DTwP in females.

The above are hypotheses and are speculative and require validation, preferably in prospective studies. However they do provide plausible immunological explanations for the observed beneficial and non-specific effects of MV and DTwP vaccines. It is important to note that whatever differences were observed at both the genetic and protein levels did not interfere with the infants' ability to generate protective antibodies to the vaccines administered.

## **8.3 Limitations of the study**

### **8.3.1 Multiplex vaccine antibody assays**

Although not a limitation, we have to point out that at the time of this study, the DTx, TTx and PTx antibody measurement by multiplex assay was not available in The Gambia and was therefore carried out by a collaborating laboratory at RIVM, Holland. However this

collaboration has subsequently led to the successful transfer of this technology to The MRC Gambia unit where this assay is now available.

A correlate of protection for pertussis toxoid antibody levels is yet to be established, and we therefore set an arbitrary cut-off value, which resulted in many of the children not achieving protective titres after vaccination. The significance of this finding is therefore not known but would suggest that the chosen cut off was too high since DTx and TTx titres were not similarly affected.

We were unable to characterize the antibody effects further due to the limited amount of plasma available. We therefore were unable to analyze for effects of sex or vaccine group on different IgG isotypes, or look at more functional aspects of the antibodies such as avidity.

### **8.3.2 Transcriptional analysis and bioinformatics**

The technology for the transcriptional profile analysis was not available in The Gambia and therefore a collaboration was established with the Division of Pathway Medicine at the University of Edinburgh. The RNA was extracted in The Gambia and then sent to Edinburgh where the assays and bioinformatics analysis were carried out. I had the opportunity to travel to Edinburgh to learn how the assays were done, but it was well outside the scope of this thesis for me to carry out all the assays and analyze the data. It is now evident that the early innate transcriptional signatures occur within a week of vaccination, and thus our 4 week post-vaccination signatures were weak and differential gene expression pathways could not be generated for all groups.

### **8.3.3 Innate cell phenotyping**

Due to the limitation in the number of markers we could use for the ICS panel, we could not include CD3 or other NK cell markers. The CD56 marker was thus used to define several

innate cell populations (NK cells, ILN-ILC1, NK T cells and  $\gamma\delta$ T cells) and ideally we would have liked to further delineate the innate cell source of the cytokines.

#### **8.3.4 Single post-vaccination time point**

The 4 week time point was chosen for the post-vaccination bleed due to the sheer logistics of having 300 children being followed up with pre- and post-vaccination bleeds. This design provides no information on the dynamics of the post-vaccination immune profile over time, nor how these dynamics might vary by vaccine group. More focused smaller time course dynamic studies would be required to test this further.

#### **8.3.5 *In vitro* culture time profiles**

All cells were cultured in overnight assays for logistic reasons, in addition to the fact that we showed in preliminary assays that we got a signal for the antigens and cytokines being tested. However, the memory / recall responses were generally low level and culture for longer time periods would have detected more robust T cell responses, particularly to the vaccine antigens.

#### **8.3.6 Sample size**

The sample size of our study was not large enough to have disease incidence or death as endpoints. With multiple assays, antigens and cytokines being tested the issue of deriving significant values that are not actually significant increases, and yet correcting for multiple testing may casue us to remove results that are significant.

### **8.4 Future directions**

To our knowledge, this is the first randomized study that has provided plausible immunological explanations for the non-specific and sex differential effects of measles and DTwP vaccination. The results have provided us with new hypotheses that can be tested and validated in prospective studies to see if they predict beneficial or deleterious outcomes in children. Samples have been collected from thousands of children in

prospective randomized trials in Guinea-Bissau, and we propose to use these samples to validate whether particular biomarker patterns predict disease incidence, hospitalization and death. Correlation of expression networks with soluble cytokine data in future studies will possibly enhance the chances of identifying these biomarker patterns.

Ideally we would like to be able to define an immune risk phenotype shortly after vaccination, which might then be used in vaccine studies. Furthermore, we would hope to define what constitutes a beneficial non-specific vaccine response with the aim of mimicking it in the future using vaccines and adjuvants.

It would be interesting to collect RNA at earlier time points (i.e. 24 hours and up to a week) from vaccinated children in order to see whether any key RNA signatures emerge in the different vaccine groups and sexes. This would provide further insights into the mechanisms of these non-specific effects, and possibly early gene signatures could be used as a screening tool for heterologous effects in vaccine trials.

In its recent systematic review, SAGE concluded that the published evidence for a deleterious effect of DTwP are still not convincing ((WHO), 2014). The immunosuppressive effects of the DTwP vaccine suggested by our data, particularly in females, therefore warrants further investigation in order to confirm it and assess whether it might be responsible for the epidemiological findings.

It might also be important to study other commonly administered vaccines for non-specific effects, and for vaccine interactions for combinations that are frequently given at the same time. This will also be apply to novel vaccines, and these effects should be considered when introducing new vaccines into existing vaccine schedules. It would also be interesting to re-visit existing immunological data sets from vaccine trials and analyze them for sex differences.

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## **APPENDIX I: MV/DTP STUDY (SCC1085)**

### **SUBJECT INFORMATION SHEET**

We would like to thank you for taking the time to consider being in this study. This form will explain the study to you.

#### **Vaccines in The Gambia**

Vaccines given in the first year of life protect your child from diseases that they might otherwise become seriously ill with, or even die from. Vaccines work by teaching your immune system to recognise and fight an infection the next time you come into contact with it. All children in The Gambia are recommended to receive routine vaccinations in the form of the Expanded Programme on Immunization (EPI). This vaccination programme is very important for the health of your child and has saved millions of lives worldwide since it was introduced.

The Gambian EPI includes having doses of the diphtheria, tetanus, pertussis combined vaccine (DTP) at 2, 3 and 4 months of age, and measles vaccine at 9 months of age. Sometimes it is not possible to have vaccines on time for reasons such as your child is unwell, or you are unable to make it to clinic. If this happens the missed vaccines may be given when you next attend. For this reason vaccines are sometimes given together or in the wrong order. For example the 3<sup>rd</sup> dose of DTP is often given with measles vaccine when the child present at 9 months of age.

#### **What is the aim of the study?**

There is now evidence that measles vaccine and DTP interact with one another if given together. This is being investigated in big studies in Guinea Bissau that we are involved in. If it is true that they interact then it may not be a good idea to give them together. We want to study in detail how they interact and how this effects the immune response of your child to infections.

### **What happens if you agree to participate?**

We will select an envelope to decide whether to give the 3<sup>rd</sup> dose of DTP at 4 months as normal or whether to give it at 9 months. The group that get DTP3 at 9 months will either have measles vaccine at 9 months with DTP3 or at 11 months of age. At 11 months all children will be given yellow fever and polio vaccines and those that have not had measles vaccine will be given it. Thus by 11 months of age your child will have received all those vaccines recommended for the 1<sup>st</sup> year of life. Your child's immunity to diphtheria, tetanus and pertussis should not be compromised by delaying the 3<sup>rd</sup> dose since 2 doses are adequate to provide protection. The delay of yellow fever and measles vaccines to 11 months is unlikely to cause an infection since the chance of your child encountering one of these diseases in the intervening two months is very low. Your child will have received polio vaccine at birth, 1, 2 and 3 months as usual, which is sufficient to provide protection, thus giving a 5<sup>th</sup> dose at 11 months is not likely to cause any problems.

We will need to take a 5 mL blood sample (1 teaspoon of blood) on 4 occasions over the study period in order to see what is happening to your child's immune system. Blood will be taken at 9 months of age, 9 ½ or 10 months (to be confirmed), 16 months and 18½ or 19 months (to be confirmed). We will look at how your child's blood responds to infections and vaccines including measles, tetanus and tuberculosis. This should help us understand if the vaccines interact and effect the immune response to other infections. We will extract nuclear material (RNA and DNA) from the blood cells and store it in order to look at the inherited genetic response to infection. We will also store serum (part of the blood that does not contain red cells) for later analysis for factors that might explain these effects. We will take a urine sample each time your child has blood taken to test for infection with a virus called cytomegalovirus (CMV).

If your child is unwell when they present for vaccination or blood sampling then the vaccine / blood sample will not be taken. You may be given an appointment for a later date. There are no risks involved with taking a teaspoon of blood from your child.

### **Your rights**

Your child will receive all their EPI vaccines at Sukuta Health Centre if they agree to participate in this study. They will also receive free health care available at Sukuta Health Centre for the duration of the study. We will compensate your travel costs to the clinic during this study. Should your child require health care outside of the Sukuta Health Centre for any reason the MRC will not be responsible.

The blood will not be used for any other study without your consent. All information that you give us will be treated as confidential. You are free to leave the study at any time you wish without giving a reason for doing so. This will not affect your access to normal medical care.

### **MRC Contacts**

Dr Katie Flanagan

MRC, Fajara

Office 4495442/3 ext 5003

Dr Jane Adetifa

Sukuta Health Centre

If you are willing to help us with this study then please sign or fingerprint the consent form.

THANK YOU.

**APPENDIX II: MV/DTP STUDY (SCC1085)**

**CONSENT FORM**

The information sheet has been read to me and I understand it / I have read and understood the information sheet.I understand what participation in the study means for me and my child.I understand that the information regarding my child that is collected in the course of this study will remain confidential.I understand that laboratory tests will be done on the blood / urine samples that my child provides, and that part of the blood will be stored for possible future tests, including genetic tests related to immunity to vaccines.

I understand that if my child gets sick during the study period, he/she can go to Sukuta clinic, and be examined and treated for free.I understand that I am free to take part in the study or refuse, and that I can withdraw my child from the study at any time, and without giving any reason. Deciding not to take part or to withdraw from the study will not affect the care that my child is normally entitled to.I have had a chance to ask questions and have them answered.

Signature or thumb print of mother / father:

\_\_\_\_\_

Name of child\_\_\_\_\_

Study No MV\_DTP |\_|\_|\_|

.....

This form has been read by / I have read the above to

\_\_\_\_\_ (write name of parent)

in a language that he/she understands. I believe that he/she has understood what I explained and that he/she has freely agreed for their child to take part in the study.

Signature of field worker:

\_\_\_\_\_

Name of field worker:

\_\_\_\_\_

Date: |\_|\_| / |\_|\_| / |\_|\_|\_|\_|